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(54) DNA sequence coding for a plant toxin of the ricin type, or a portion thereof.

(57) DNA comprising a nucleotide sequence coding for at least a portion of the precursor polypeptide of ricin or a similar plant toxin. Recombinant DNA molecules containing such DNA as an insert, and genetically-modified host microorganisms containing such recombinant DNA molecules.

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DNA

This invention relates to DNA comprising a nucleotide sequence coding for at least a portion of a polypeptide which is a plant toxin of the ricin type, as hereinafter defined. It also relates to recombinant DNA molecules comprising a DNA sequence which codes for a polypeptide which is or is closely related to a natural plant toxin of the ricin type. Ricin, and also other plant toxins such as abrin, modeccin and viscumin, consist of two polypeptide chains (known as the A and B chains) linked by a disulphide bridge, one chain (the A chain) being primarily responsible for the cytotoxic and the other chain (the B chain) having sites enabling the molecule to bind to cell surfaces. Ricin is produced in the plant Ricinis communis (also known as the castor bean plant) via a precursor protein known as "preproricin".

Preproricin comprises a single polypeptide chain which includes a leader sequence. The leader sequence is subsequently removed in the organism to give proricin which is then cleaved to eliminate a linker region and joined by a disulphide bond to from the mature protein.

The toxicity of ricin-type toxins operat s in three phases: (1) binding to the cell surface via the B chain; (2) penetration of at least the A chain into the cytosol, and (3) inhibition of protein synthesis through the A chain attacking the 60S subunits of the ribosomes. Thus, separated A and B chains are essentially non-toxic, the inherently toxic A chain lacking the ability to bind to cell surfaces in the absence of the B chain.

It is also known that in ricin-type toxins the B chain binds to cell surfaces by virtue of galactose recognition sites, which react with glycoproteins or glycolipids exposed at the cell surface.

It has already been suggested that the toxicity of the ricin A chain might be exploited in anti-tumour therapy, by replacing the indiscriminately-binding B chain with a different carrier component having the ability to bind only to tumour cells. Thus, various immunotoxins have already been prepared, consisting of a conjugate of whole ricin or a separated natural ricin A chain and a tumour-specific monoclonal antibody. Although these known conjugates are of considerable potential in themselves, there is scope for improvement.

One problem with the known conjugates arises from a structural feature of the A chain from natural ricin. It is known that the natural ricin A chain becomes N-glycosylated during its synthesis, by enzymes present

in Ricinus c lls, and it is thought that the resulting sugar moieties are capable of non-sp cific int ractions with cell surfaces. Thus, it appears that the known A chain conjugates are capable of a certain amount of binding with non target cells, even in the absence of the natural B chain, thus increasing the toxicity of such immunotoxins towards non target cells.

Another problem with the known ricin A chain conjugates stems from the fact that the B chain seems to have an important secondary function in that it somehow assists in the intoxication process, apart from its primary function in binding the ricin molecule to the cell surface. This secondary function is lost if the B chain is replaced by a different carrier component such as a monoclonal antibody.

If it were possible to prevent interactions between the cell surface via the A chain sugar moieties, whilst preserving the secondary toxicity-increasing function of the B chain, the toxicity of a whole ricin antibody conjugate towards normal cells could be reduced, and towards target cells could be increased, thus improving the therapeutic index of the immunotoxin. It is also known that the natural ricin B chain is N-glycosylated and the B chain sugar moieties may also contribute to non specific interactions, Also, the sugar moieties in both chains enable the ricin molecule to be sequestrated by reticuloendothelial cells in the liver, and so would lead to the

rapid excretion from the system of a drug based on a part or the whole of the ricin molecule in which such sugar moieties were still present.

Attempts to remove all the sugar moieties from natural ricin by chemical or enzymatic methods have so far failed. Nevertheless the major obstacle confronting the use of known whole ricin-antibody conjugates is the presence of two galactose binding sites in the ricin B chain. These B chain galactose binding sites are primarily responsible for the non-specific cellular interactions of current whole ricin-antibody conjugates, particularly when used in vivo. Their presence in the natural toxin clearly eliminates or reduces the targeting specifity conferred by the antibody.

An improved immunotoxin based on ricin or another plant toxin of the ricin type, not suffering from these problems, could consist of a whole toxin molecule modified so that it is not N-glycosylated, and so that the B chain has no galactose recognition sites, but retains its secondary intoxication-promoting properties, coupled to a carrier moeity which delivers the toxin to the target cells. This could be a tumour-specific or cell/tissue specific vehicle such as a suitable monoclonal antibody.

Our research which has so far been concentrated on ricin itself, has indicated that the assembly of ricin (and the related agglutinin which consists of

two ricin-like molecules with slightly modified A and B chains) does not involve the separate synthesis of the A and B chains as the products of distinct mRNA's, but rather the initial formation of a single polypeptide precursor containing both the A chain and B chain sequences. This is thought to apply in the case of other toxins of the same type.

This invention is based on the idea of preparing a genetically-engineered microorganism capable of expressing a molecule of a toxin of the ricin type, as defined above,

or alternatively part of such a toxin molecule, or a precursor of such a molecule (which could be converted to the toxin molecule itself) which toxin molecule could be modified as suggested above and could be used to construct an effective toxin conjugate by combining it with a tumour-specific or cell/tissue specific monoclonal antibody or other carrier moiety, such as a hormone or lectin.

The fact that ricin is formed via a precursor polypeptide will enable a cell system to be constructed by known techniques which expresses a ricin precursor. The ricin precursor product could then be chemically or enzymatically converted to the desired modified ricin. An analagous technique could be used in the case of other ricin-type toxins as herein defined. An alternative technique would be to divide from the DNA sequence that codes

for the precursor two sequences which code separately for the A and B chains, to insert these separated sequences into different cloning vehicles and to insert the resulting recombinant DNA molecules into separate host microorganisms. One such host would then express the A chain polypeptide sequence and the other the B chain polypeptide sequence. These sequences could then be combined to form the desired modified ricin molecule. This technique could obviously also be used for any ricin-type toxins in which the A and B chains are encoded by distinct mRNA gene pools. This latter approach would be preferred on safety grounds, in that separate and therefore non-toxic A and B chains would be expressed.

According to one aspect of the invention we provide a biologically pure and homogeneous sample of DNA comprising a nucleotide sequence coding for at least a portion of a precursor of a ricin-type toxin polypeptide, or mutants thereof.

Said portion preferably comprises the A chain or the B chain of the mature protein.

More specifically, we provide a sample of DNA including at least a substantial portion of any of the following DNA sequences, which sample is biologically-pure:

ATG TAT GCA GTG GCA ACA TGG CTT

TGT TTT GGA TCC ACC TCA GGG TGG TCT TTC ACA TTA GAG GAT AAC AAC ATA

TTC CCC AAA CAA TAC CCA ATT ATA AAC TTT ACC ACA GCG GGT GCC ACT GTG

CAA AGC TAC ACA AAC TTT ATC AGA GCT GTT CGC GGT CGT TTA ACA ACT GGA

GCT GAT GTG AGA CAT GAT ATA CCA GTG TTG CCA AAC AGA GTT GGT TTG CCT

ATA AAC CAA CGG TTT ATT TTA GTT GAA CTC TCA AAT CAT GCA GAG CTT TCT

GTT ACA TTA GCC CTG GAT GTC ACC AAT GCA TAT GTG GTC GGC TAC CGT GCT

GGA AAT AGC GCA TAT TTC TTT CAT CCT GAC AAT CAG GAA GAT GCA GAA GCA

ATC ACT CAT CTT TTC ACT GAT GTT CAA AAT CGA TAT ACA TTC GCC TTT GGT

Page 7a follows...

GGI AAI TAT GAT AGA CTT GAA CAA CTI GCI GGT AAI CTG AGA GAA AAT ATC

GAG TTG GGA AAI GGT CCA CTA GAG GAG GCI AIC TCA GCG CTI TAT TAT TAC

AGT ACT GGT GGC ACT CAG CTT CCA ACI CTG GCT CGI TCC TTT ATA ATT TGC

ATC CAA ATG ATT TCA GAA GCA GCA AGA TTC CAA TAT ATT GAG GGA GAA ATG

CGC ACG AGA ATI AGG TAC AAC CGG AGA TCI GCA CCA GAI CCI AGC GIA AII

ACA CTI GAG AAI AGI TGG GGG AGA CTI TCC ACT GCA ATT CAA GAG TCI AAC

CAA GGA GCC TTT GCI AGI CCA ATT CAA CTG CAA AGA CGI AAI GGI TCC AAA

TTC AGI GTG TAC GAT GTG AGI ATA TIA AIC CCI AIC AIA GCI CTC ATG GIG

TAI AGA TGC GCA CCT CCA CCA TCG TCA CAG TTI TCI TTG CTI ATA AGG CCA

Page 7b follows...

GTG GTA CCA AAT TTT AAT GCT GAT GTT TGT ATG GAT CCT GAG CCC ATA GTG

CGT ATC GTA GGT CGA AAT GGT CTA TGT GTT GAT GTT AGG GAT GGA AGA TTC

CAC AAC GGA AAC GCA ATA CAG TTG TGG CCA TGC AAG TCT AAT ACA GAT GCA

AAT CAG CTC TGG ACT TTG AAA AGA GAC AAT ACT ATT CGA TCT AAT GGA AAG

5. TGT TTA ACT ACT TAC GGG TAC AGT CCG GGA GTC TAT GTG ATG ATC TAT GAT

TGC AAT ACT GCT GCA ACT GAT GCC ACC CGC TGG CAA ATA TGG GAT

AAT GGA ACC ATC ATA AAT CCC AGA TCT AGT CTA GTT TTA GCA GCG ACA TCA

GGG AAC AGT GGT ACC ACA CTT ACG GTG CAA ACC AAC ATT TAT GCC GTT AGT

CAA GGT TGG CTT CCT ACT AAT AAT ACA CAA CCT TTT GTT ACA ACC ATT GTT

Page 7c follows...

GGG CTA TAT GGI CTG TGC TTG CAA GCA AAT AGT GGA CAA GTA TGG ATA GAG

GAC TGT AGC AGT GAA AAG GCT GAA CAA CAG TGG GCT CTT TAT GCA GAT GGT

TCA ATA CGT CCT CAG CAA AAC CGA GAT AAT TGC CTT ACA AGT GAT TCT AAT

ATA CGG GAA ACA GTT GTT AAG ATC CTC TCT TGT GGC CCT GCA TCC TCT GGC

5. CAA CGA TGG ATG TTC AAG AAT GAT GGA ACC ATT TTA AAT TTG TAT AGT GGA

TTG GTG TTA GAT GTG AGG CGA TCG GAT CCG AGC CTT AAA CAA ATC ATT CTT

TAC CCT CTC CAT GGT GAC CCA AAC CAA ATA TGG TTA CCA TTA TTT

or

ATG TAT GCA GTG GCA ACA TGG CTT

TGT TTT GGA TCC ACC TCA GGG TGG TCT TTC ACA TTA GAG GAT AAC AAC ATA

TTC CCC AAA CAA TAC CCA ATT ATA AAC TTT ACC ACA GCG GGT GCC ACT GTG

CAA AGC TAC ACA AAC TTT ATC AGA GCT GTT CGC GGT CGT TTA ACA ACT GGA

GCT GAT GTG AGA CAT GAT ATA CCA GTG TTG CCA AAC AGA GTT GGT TTG CCT

ATA AAC CAA CGG TTT ATT TTA GTT GAA CTC TCA AAT CAT GCA GAG CTT TCT

GTT ACA TTA GCC CTG GAT GTC ACC AAT GCA TAT GTG GTC GGC TAC CGT GCT

GGA AAT AGC GCA TAT TTC TTT CAT CCT GAC AAT CAG GAA GAT GCA GAA GCA

ATC ACT CAT CTT TTC ACT GAT GTT CAA AAT CGA TAT ACA TTC GCC TTT GGT

Page 8a follows...

GGT AAT TAT GAT AGA CTT GAA CAA CTT GCT GGT AAT CTG AGA GAA AAT ATC

GAG TTG GGA AAT GGT CCA CTA GAG GAG GCT ATC TCA GCG CTT TAT TAT TAC

AGT ACT GGT GGC ACT CAG CTT CCA ACT CTG GCT CGT TCC TTT ATA ATT TGC

ATC CAA ATG ATT TCA GAA GCA GCA AGA TTC CAA TAT ATT GAG GGA GAA ATG

5. CGC ACG AGA ATT AGG TAC AAC CGG AGA TCT GCA CCA GAT CCT AGC GTA ATT

ACA CTT GAG AAT AGT TGG GGG AGA CTT TCC ACT GCA ATT CAA GAG TCT AAC

CAA GGA GCC TTT GCT AGT CCA ATT CAA CTG CAA AGA CGT AAT GGT TCC AAA

TTC AGT GTG TAC GAT GTG AGT ATA TTA ATC CCT ATC ATA GCT CTC ATG GTG

TAT AGA TGC GCA CCT CCA CCA TCG TCA CAG TTT

or

Page 8b f llows

GCT GAT GTT TGT ATG GAT CCT GAG CCC ATA GTG

CGT ATC GTA GGT CGA AAT GGT CTA TGT GTT GAT GTT AGG GAT GGA AGA TTC

CAC AAC GGA AAC GCA ATA CAG TTG TGG CCA TGC AAG TCT AAT ACA GAT GCA

AAT CAG CTC TGG ACT TTG AAA AGA GAC AAT ACT ATT CGA TCT AAT GGA AAG

5. TGT TTA ACT ACT TAC GGG TAC AGT CCG GGA GTC TAT GTG ATG ATC TAT GAT

TGC AAT ACT GCT GCA ACT GAT GCC ACC CGC TGG CAA ATA TGG GAT

AAT GGA ACC ATC ATA AAT CCC AGA TCT AGT CTA GTT TTA GCA GCG ACA TCA

GGG AAC AGT GGT ACC ACA CTT ACG GTG CAA ACC AAC ATT TAT GCC GTT AGT

CAA GGT TGG CTT CCT ACT AAT AAT ACA CAA CCT TTT GTT ACA ACC ATT GTT

Page 8c follows...

GGG CTA TAT GGT CTG TGC TTG CAA GCA AAT AGT GGA CAA GTA TGG ATA GAG

GAC TGT AGC AGT GAA AAG GCT GAA CAA CAG TGG GCT CTT TAT GCA GAT GGT

TCA ATA CGT CCT CAG CAA AAC CGA GAT AAT TGC CTT ACA AGT GAT TCT AAT

ATA CGG GAA ACA GTT GTT AAG ATC CTC TCT TGT GGC CCT GCA TCC TCT GGC

CAA CGA TGG ATG TTC AAG AAT GAT GGA ACC ATT TTA AAT TTG TAT AGT GGA

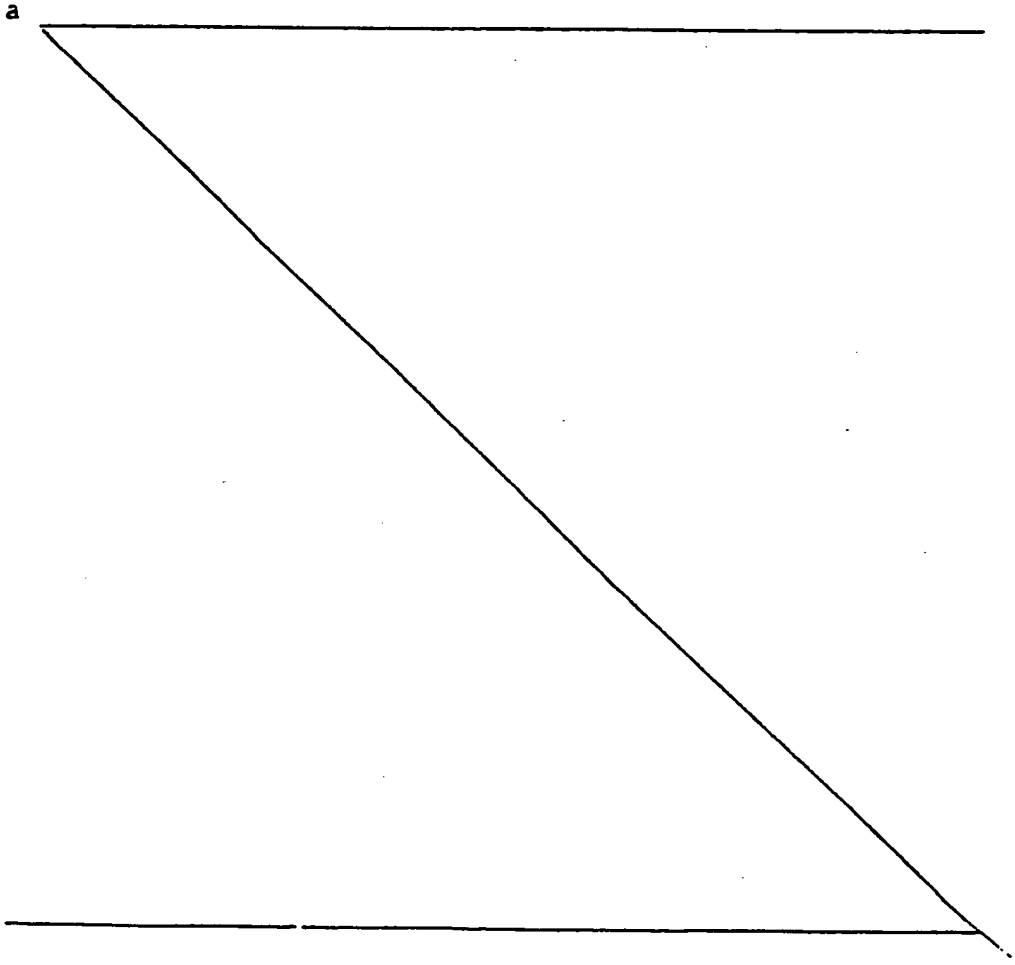
TTG GTG TTA GAT GTG AGG CGA TCG GAT CCG AGC CTT AAA CAA ATC ATT CTT

TAC CCT CTC CAT GGT GAC CCA AAC CAA ATA TGG TTA CCA TTA TTT

or at least a portion of a nucleotid sequence which is equivalent thereto by virtue of degeneracy of the genetic code.

According to another aspect of this invention we provide a recombinant DNA molecule comprising a DNA sequence which codes for a polypeptide sequence present in a plant toxin of the ricin type, as defined herein.

More specifically, we provide a recombinant DNA molecule containing a DNA sequence which codes for a



A and B chain precursor polypeptide of a plant toxin of the ricin typ .

Alternatively, we provide a recombinant DNA molecule containing a DNA sequence coding for at least a portion of either the A chain or the B chain of a plant toxin of the ricin type.

According to a further aspect of the present invention we provide a genetically modified host micro-organism which contains a recombinant DNA molecule as hereinbefore defined.

In the recombinant DNA molecules according to the present invention, the nucleotide sequence coding for the B chain may be modified to eliminate or inactivate the galactose binding sites, and the precursor polypeptide and hence in the mature protein, to eliminate or inactivate the galactose binding sites, and the sequences encoding ther signals for N-glycosylation may also be modified to render them ineffective or to eliminate them. Examples of techniques which may prove useful are deletion or oligonu cleotide mediated mutagenesis.

The host organism may be a plant cell or an animal cell or preferably a microorganism.

The microorganism may be a prokaryote or a eukaryote. As examples of prokaryotes may be mentioned Gram-negative bacteria, e.g. E.coli., Methylophilus methylotrophus and Alcaligenes eutrophus; and Gram-positiv

bact ria, e.g. Streptomyces, Bacillus subtilis and Arthrobacter. As examples f eukaryot s may be mentioned yeasts, for example Saccharomyces cerevisiae.

The recombinant DNA molecules may comprise a cloning vector such as a plasmid or phage vector into which has been inserted the DNA sequence coding for at least a portion of a precursor polypeptide, or at least a portion of either the A chain or the B chain, of a ricin-type plant toxin.

The cloning vector is preferably a plasmid although we do not exclude the possibility that it may be a phage vector. The plasmid may be a naturally-occurring plasmid or preferably a composite derived from fragments of other plasmids. Where a composite plasmid is employed it preferably contains promoter sequences which improve expression of the ricin gene.

Examples of suitable plasmids which may be used as cloning vehicles are inter alia for Gram-negative bacteria: pBR322, pAT153, pUC8, pGSS15 and pMB9; for Gram-positive bacteria: pVC6; and for S.cerevisiae: pMA91, pMA230, YRp7, pLC544 and YEp6. The vector will be selected to be suitable for the particular host envisaged.

We also provide a method of obtaining a recombinant DNA molecule which comprises preparing a double-stranded DNA sequence which codes for a polypeptide sequence present in a plant toxin of the ricin type and inserting said double-stranded DNA sequence into a cloning vector.

More specifically such a method may comprise isolating the mRNA which codes for the ricin A and B chain precursor polypeptide, synthesising single-stranded cDNA from said mRNA using reverse transcriptase and a suitable primer, assembling the second DNA strand onto the template formed by said first strand by means of DNA polymerase followed by S1 nuclease, and inserting the resulting double-stranded cDNA into a cloning vector.

Alternatively, the cDNA assembled from the mRNA may be cut into separate portions which code respectively for separate portions of the ricin molecule, for example for the A and B chains, which portions are then inserted into separate cloning vectors.

As stated above the cloning vector is preferably a plasmid such as pBR322, pAT153, of pUC8, and this may be cut open by means of the restriction endonuclease Pst I, and may be tailed with oligo (dG), and annealed with the double-stranded cDNA which has been tailed with oligo (dC).

We also provide a method of producing a modified transformed host by introducing into a suitable host microorganism a recombinant DNA molecule according to this invention.

The microorganism used as the host for cloning is preferably a Gram-negative bacterium and more preferably E. coli.

After cloning, the DNA sequence coding for the ricin precursor (or the precursor of another ricin-type

toxin which is f rmed from a precursor) may be removed from the host cloning vector. It may then be divided into two portions which code for separate regions of the toxin molecule, for example the A and B chains, these portions introduced into separate second cloning vectors and new hosts modified with each of the resulting new recombinant DNA molecules. Alternatively it may be introduced whole into a second cloning vector. The second cloning vectors comprise suitable promoter sequences and the position and direction of insertion of the whole coding sequence or portion thereof into the second cloning vector are such that on introducing the new recombinant DNA molecules into suitable host microorganisms, e.g. E. coli or S.cerevisiae, expression of the desired gene sequence is obtained.

The preparation of a transformed host containing a DNA sequence which codes for the ricin A and B chain precursor polypeptide will now be described by way of example, first in general terms and then in detail. This process is summarised in the accompanying diagram.

Firstly, the mRNA encoding this precursor was enriched in known manner by sucrose density gradient centrifugation. The corresponding cDNA was assembled in single-strand form onto this mRNA in known manner using the enzyme reverse transcriptase, a growing point having first been provided on the mRNA using oligo (dT) as primer which binds onto the polyadenylated 3'-terminus

f th mRNA. The imm diate product of this reaction is a DNA-RNA hybrid. The RNA strand is removed by hydrolysis, leaving the single-strand DNA intact. This is converted to the double stranded form using the enzyme DNA polymerase in the presence of free nucleotides, which results in a hairpin-shaped molecule, the curved end of which is then removed by the single-strand specific nuclease S1. The resulting double strand cDNA is then tailed with oligo (dC)⁄, using terminal transferase size fractionated to remove small molecules, or vice versa and annealed with the pBR322 or pAT153 vector which has been cut open with Pst I and tailed with oligo (dG)⁄, using terminal transferase the cytosine tails on the DNA base pairing with the guanine tails on the vector.

The resulting chimaeric plasmids containing the DNA segment coding for the ricin precursor polypeptide were then used to transform E-coli DH1 cells, and the presence of the chimaeric plasmid was ensured by selecting cells displaying tetracycline resistance and ampicillin sensitivity. Over 1600 Tet^r, Amp^s clones were obtained. Colonies derived from each clone were transferred to nitrocellulose filters and clones containing the desired DNA sequence identified using a 32P-end labelled 20 mer oligonucleotide probe, having the DNA sequence $ACCTACAA^{A}_{G}TT^{C}_{T}TT^{A}_{G}CT^{A}_{G}CC$ which hybridises to DNA containing the complementary sequence $TGGATGTT^{T}_{C}AA^{G}_{A}AA^{T}_{C}GA^{T}_{C}GG$. As the ricin precursor

polypeptide have been found to contain the amino sequence - Trp-Met-Phe-Lys-Asn-Asp-Gly- the DNA sequence responsible for this is known from the genetic code to be the latter mentioned above.

Using appropriate hybridisation and wash conditions, e.g. as described by Singer-Sam et al in (1983) Proc. Natl.Acad.Sci.(U.S.A.), Vol.80 pp 802-806, 80 clones were selected as positively containing the desired DNA sequence, and of these, the eight largest in the plasmid pBR322 have been initially chosen for further characterisation. Their relationship to the castor bean lectin precursor polypeptides has been confirmed using the hybrid release translation assay. Of the eight clones mentioned above, four, respectively with 1614, 1950, 1059 and 1020 base pairs, have been selected for sequencing.

In detail, the transformed host was prepared as follows:

A. cDNA synthesis

1. mRNA extraction and fractionation

100-200 g of ripening Ricinus seeds were frozen and ground to a powder in liquid nitrogen, and homogenised in a Waring blender for 1 - 2 minutes in 50 mM tris-HCl pH 9, 150 mM NaCl, 5 mM EDTA and 5% SDS. The homogenate was extracted with an equal volume of phenol:chloroform (1:1) and the phases were separated by centrifugation. The rganic phase and residue

were reextracted with 0.5 volume f 20 mM tris-HCl pH 9.0, 2 mM EDTA and the resultant aque us phase was combined with the original one. The total aqueous phase was reextracted repeatedly with equal volumes of phenol:chloroform until no material was present at the interface. RNA was precipitated by the addition of 2 volumes of cold ethanol after making the solution 200 mM in NaCl.

After overnight precipitation at - 20°C the RNA was centrifuged at 10,000 rpm for 30 minutes in an MSE 18 or MSE 21 centrifuge; the pellet was then washed repeatedly in 3 M NaAc pH 5.5 until no polysaccharide was detectable in the supernatant by ethanol precipitation. The final pellet was dissolved in 300 mM NaCl and precipitated as above.

mRNA molecules bearing poly(A) tails were extracted by affinity chromatography on oligo(dT)-cellulose: after hybridisation at room temperature for 30 min. in 400 mM NaCl, 20 mM tris-HCl pH 7.6, 0.2% SDS, the beads were pelleted and washed three times in the above buffer and two times in 200 mM NaCl, 20 mM tris-HCl pH 7.6, 0.1% SDS. The slurry was poured into a column and washed further with the last buffer until the A_{260} of the eluate reached the background level. Poly(A)-containing RNA was then eluted with 20 mM tris-HCl pH 7.6 at 50°C. The eluate was monitored with an ISCO continuous flow UV cell. Poly(A)-containing RNA was precipitated overnight from 200 mM NaCl by the addition of 2 volumes of cold ethanol at - 20°C and was then washed

thre times with 70% ethanol, and redissolved in 10 mM tris-HCl pH 7.0 to approximately 1 µg/µl.

The mRNA was heated for 2 min. at 65° C and quench cooled. Approximately 400 µg of poly(A)$^+$ RNA was layered on top of a 10 - 30% ribonuclease-free sucrose (Sigma) density gradient in 100 mM tris-HCl pH 7.5, 0.5% SDS, 1 mM EDTA, and centrifuged in a Beckman L5-65B centrifuge using an SW27 rotor at 25,000 rpm at 17°C for 14 hours. 400 µl fractions were collected with an ISCO density gradient fractionator using the continuous flow UV cell.

Each fraction was made 200 mM in NaCl and precipitated with 2 volumes of cold ethanol by freeze-thawing in liquid nitrogen three times, and recovered by centrifugation in an Eppendorf microcentrifuge for 30 minutes at 4° C, washed once with 70% ethanol, and redissolved in 10 µl of 10 mM tris-HCl pH 7.0. An aliquot from each fraction (1 µl) was translated in a reticulocyte lysate cell free system and the lectin precursor immunoprecipitated to identify the fraction enriched for lectin mRNA.

2. First strand synthesis

Fractionated poly(A)$^+$ RNA was reverse transcribed at 0.5 µg/µl in the presence of 50 mM tris-HCl pH 8.3, 10 mM $MgCl_2$, 100 mM KCl, 1 mM of dATP, dTTP and dGTP, 250 µM dCTP, 0.06 µg/µl olig $(dT)_{12-18}$, 10 mM DTT and 0.4 units/µl of reverse transcriptase

fr m avian myeloblast sis virus. (3H)dCTP or α-(^{32}P)dCTP were included in the reaction as appropriate.

The reaction mixture was incubated at 42 C for 45 minutes, at which point an equal volume of 5 mM tris-HCl pH 8.3, 5 mM DTT, 250 µM dCTP was added along with the same amount of enzyme as previously. The reaction was incubated for a further 45 minutes at 45°C and terminated by freezing. Aliquots were analysed on 1% denaturing agarose gels along with the products of the second strand and S_1 nuclease reactions.

3. Second strand synthesis

mRNA-cDNA hybrids were denatured by boiling the first strand reaction for 3 minutes and cooling rapidly. After pelleting insoluble material in the Eppendorf microfuge for 2 minutes the supernatant was transferred to a fresh chilled tube. For the standard reaction, reagents were added as follows, ignoring elements already present: dATP, dGTP and dTTP to 100 µM, Hepes-KOH pH 6.9 to 105 mM, KCl to 92 mM, dCTP, labelled as appropriate, to 80 µM, and 0.1 units/µl of DNA polymerase. The reaction was allowed to proceed at 20°C for 6 hours, at which time cDNA was removed from the mixture by gel filtration on 1 ml columns of Bio-Gel P60 in 10 mM tris-HCl pH 7.6, 20 mM NaCl, 1 mM EDTA. Fracti ns were monitored by Cerenkov or liquid scintillation counting,

and peak excluded fractions were pooled and precipitated from 0.3 M NaAc pH 6 by the addition f 2 volumes of cold ethanol. Precipitates were recovered by centrifugation in the Eppendorf micro-centrifuge for 30 minutes in the cold, and dissolved in water to about 2.5 µg/µl of RNA - equivalent material.

4. S_1 nuclease digestion

Single-stranded regions of double-stranded cDNA were digested with S_1 nuclease from Aspergillus oryzae, in the presence of 300 mM NaCl, 30 mM NaAc pH 4.5, 3 mM $ZnCl_2$. The reaction was incubated for 15 minutes at 37°C and then for 15 minutes at 15°C, and was terminated by the addition of tris-HCl pH 7.6 to 130 mM and EDTA to 10 mM; it was then extracted with an equal volume of phenol:chloroform: isoamyl alcohol (25:24:1) and precipitated from 300 mM NaAc pH 6 with 2 volumes of cold ethanol. The precipitate was dissolved in 10 mM tris-HCl pH 8, 0.1 mM EDTA to 0.25 µg/µl RNA equivalent.

5. Addition of homopolymer tails to DNA

Double-stranded DNA was tailed using terminal transferase with dCTP at 0.001 - 0.01 µg/µl in the presence of 140 mM potassium cacodylate pH 7.6, 30 mM tris base, 0.1 mM DTT,

1 mM $CoCl_2$ and (^3H) or (^{32}P) - labelled dCTP in 75 - 150 fold excess over 3' termini. The reaction was carried out at 37 C for 6 minutes. The extent of incorporation of label was followed by assaying the amount of TCA - insoluble radioactivity as a proportion of the total radioactivity, counting in Bray's scintillant.

The reaction was stopped by chilling and adding EDTA to 10 mM, after which unincorporated material was removed by gel filtration as described. Tailed cDNA was precipitated as before, and dissolved in 1 M NaAc pH 8, 10 mM tris-acetate pH 8, 1 mM EDTA ready for fractionation.

PstI-cleaved pBR322 DNA was similarly treated, except that dGTP replaced dCTP.

6. Fractionation of tailed cDNA

cDNA was fractionated on 5 - 20% linear sucrose density gradients in 1 M NaAc pH 8, 10 mM tris-acetate pH 8, 1 mM EDTA, and centrifuged overnight at 39,000 rpm in an SW50.1 rotor. DNA sedimentation was checked on a parallel gradient loaded with a mixture of HinfI and PstI digests of pBR322 DNA, and fractions of this gradient were run on a 1% neutral agarose gel. Fractions from the cDNA gradient

were diluted with an equal volume of water and precipitated with 2 volumes of cold ethanol, and then pooled to give three final fractions, a large cDNA fraction (larger than 2,200 bp), an intermediate fraction (1,000 - 2,200 bp) and a fraction containing smaller cDNAs (600 - 1,000 bp). cDNA molecules smaller than 600 bp were discarded.

The three final fractions were dissolved to approximately 5 ng/µl in 150 mM RbCl, 10 mM tris-HCl pH 7.6, 0.2 mM EDTA.

B. Annealing and transformation

1. Annealing

dC-tailed cDNA was mixed with dG-tailed pBR322 or pAT153 in approximately equimolar quantities, at a concentration of 0.4 ng/µl of vector. Buffers were as described above. The mixtures were heated to 70°C for 30 minutes and then cooled overnight to room temperature, and slowly chilled to 4°C. Competent cells were added and transformed as described below.

2. Preparation of competent cells and transformation

DH1 cells* were grown in 10 ml cultures of psi broth (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 20 mM $MgCl_2$, pH adjusted to 7.6 with KOH; all bacteriological reagents from Difco), and grown at 37°C in a shaking waterbath to A_{550} = 0.3. 1 ml of this was then inoculated into 25 ml of the

* (recA1,nalA, r_k^-, m_k^+, ndol$^-$, R$^-$, relA1?)

same medium and grown to A_{550} = 0.48. The cells were then chilled on ice for 15 minutes and harvested at 5,000 rpm for 5 minutes in an MSE 21 centrifuge at 4°C. They were then resuspended in 10 ml of 100 mM RbCl, 50 mM $McCl_2$, 10 mM $CaCl_2$, 35 mM NaAc pH 5.8, 15% glycerol and kept on ice for 15 minutes.

The cells were again harvested, and resuspended in 1 ml of 10 mM RbCl, 75 mM $CaCl_2$, 10 mM MOPS - KOH pH 5.8, 15% glycerol, and kept on ice for a further 15 minutes.

100 µl of the cells thus prepared were mixed with the annealed DNA samples, and incubated on ice for 30 minutes, after which they were heat-shocked at 42°C for 90 - 120 seconds. 1 ml of psi broth was added, and the cells were grown at 37°C for 1 hour. They were then centrifuged briefly, and resuspended in 100 µl of psi broth and plate on LB plates containing 14 µg/ml tetracycline (LB is 1% tryptone, 0.5% yeast extract, 170 mM NaCl, 1.5% agar).

After 18 - 24 hours growth at 37°C, colonies were counted and spotted onto LB plates containing 33 µg/ml ampicillin to identify those transformants containing recircularised or uncut plasmids. Over 1600 Tet^r Amp^s clones wer picked and transferred

in order d arrays onto large plates of LB containing 14 µg/ml tetracyclin .

C. Screening

1. Labelling of oligonucleotide

The ricin B chain specific oligomer (20 mer) was end labelled using polynucleotide kinase. 500 ng of oligonucleotide was incubated in 50 mM tris pH 8.5, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine-HCl, 0.1 mM EDTA with 60 µCi $\gamma(^{32}p)$ ATP and 1 µl polynucleotide kinase (Boehringer) for 35 min at 37°C. The reaction was stopped by adding an equal volume of 0.6 M NH_4AC and the bulk of non incorporated γ ATP was removed by passage through a sephadex G25 column in 0.14 M NaCl, 0.02 M tris pH 7.6, 0.005 M EDTA, 0.1% SDS. The probe was stored frozen at -20°C.

2. Colony Hybridization using oligonucleotide probe

Transformants were grown on nitrocellulose filters (Schleicher & Schuell 0.45 µ) layered over LB plus tetracycline. The filters, in triplicate, were then transferred to LB-Tet plates containing 200 µg/ml chloramphenicol f r 16 h at 37°. The filters were placed colony side up on two sheets of 3 mm paper wetted with 0.5 M NaOH for 15 mins at room

temperature. Th same procedure was followed for th following two washes (1) with 1M tris pH 8.0, and (2) with 1 M tris pH 8, 1.5 M NaCl (30 mins). The filters were air dried and baked at 80°C.

Prehybridizations and hybridizations were done in double sealed polythene bags. The filters were prehybridized in 0.9 M NaCl, 0.09 M tris 7.4, 0.006 M EDTA, 0.5% NP40, 2x Denhardts, 0.2% SDS, 100 µg/ml denatured single strand salmon sperm DNA and 70 µg/ml tRNA. Prehybridization was done for 4 h at 55°C. The prehybridization buffer was then squeezed from the bag and fresh buffer added that contained 50 ng labelled probe (to give a maximum concentration of 5 ng/ml buffer). Annealing was done overnight at 37°C.

Non stringent washes were done with 6 x SSC at room temperature. The filters were washed in 4 changes of 6 x SSC over 3h. The triplicate filters were then washed at three different temperatures determined from the base composition and degree of mismatch of the probe. Using 2°C for every A or T and 4°C for every C or G in the probe the wash temperatures selected were 52°C, 56°C and 60°C. The filters were washed at the stringent temperature in 6 x SSC for 10 minutes and then dried thoroughly. The filters were exposed to X-ray film overnight.

D. Hybrid selection procedure

1. DNA binding

Plasmid DNA was purifi d from the positive clone(s) and 10-15 µg linearized with EcoR1. After phenol: chloroform extraction and ethanol precipitation the pellet was dissolved in 0.5 ml 0.1 x SSC. 0.5 ml 1 M NaOH was then added and the mixture allowed to stand for 15 mins at room temperature. 4 ml of a prechilled neutralizing solution (1.5 M NaCl, 0.25 M HCl, 0.25 M tris- Cl pH 8.0) was added and the 5 ml DNA sample sucked by vacuum through swinnies containing wetted Schleicher and Schuell 0.45 µ filter discs. 5 ml 6 x SSC was then passed through the filter(s). These were air dried and then baked for 2 h at 80°C.

2. Hybrid selection protocol

The filter(s) were placed in 5 ml bottles and prehybridized for 4 h at 41°C in 50% formamide, 0.4 M NaCl, 10 mM pipes-NaOH pH 6.4, 4 mM EDTA, 0.5 µg/ml tRNA, 10 µg/ml poly (A). The buffer was removed and the filter(s) typically hybridized overnight at 41°C in 50% formamide buffer (above) containing approx. 20 µg poly $(A)^+$ RNA from castor beans. The buffer was removed and the filters washed twice for 15 mins in each of the following: (1) 1 x SSC, 0.5% SDS at room

temperature, (2) 0.1 x SSC, 0.1% SDS at room temperature, (3) 0.1 x SSC, 0.1% SDS at 50°C, (4) 0.1 x SSC, 0.1% SDS at room temperature. The filters wer drain d and 200 ul hybrid release buffer (90% formamide, 10 mM pipes-NaOH pH 6.4, 1 mM EDTA, 0.5% SDS) added to each and mixed for 30 mins at 40°C. The buffer was removed into a fresh eppendorf and NaCl added to .2 M. The released mRNA was precipitated with ethanol, rinsed several times in 70% ethanol, dried and dissolved in 5 µl sterile water. The sample was translated in a reticulocyte lysate cell free system and the products run out directly on an SDS-polyacrylamide gel or firstly immunoprecipitated with appropriate antisera.

The DNA sequence coding for the above-mentioned ricin precursor polypeptide of two of the above-mentioned clones, referred to herein as pBRCL 6 and pBRCL 17 (RCL = Ricinus communis lectin) has now been determined by a combination of the Sanger dideoxy method (Sanger et al, 1977 - Proc. Natl. Acad. Sci. U.S.A. 74, 5463-67) and the procedure of Maxam and Gilbert (Maxam and Gilbert, 1980 - Meth. Enzym. 65, 499-560). In order to determine the sequence at the ends of each insert the inserts were excised from pBR322 with Pst I and ligated into Pst I linearized, phosphatased plasmid pUC8 (Vierra and Messing, 1982 - Gene 19, 259-268). E. Coli DHI cells were transformed by these recombinant plasmids. These new recombinant plasmids ar referred to herein as pRCL6 and pRCL17.

It is apparent that the two inserts contain a region of common sequence and that together they represent a total ricin precursor sequence. There are no nucleotide differences between the overlapping regions of the inserts in pRCL6 and pRCL17.

A new recombinant DNA molecule was then constructed that contains the complete nucleotide sequence encoding the ricin precursor polypeptide. This was achieved by isolating a fragment, 323 base pairs in length, obtained from pRCL17 by digestion with the restriction endonuclease Sau 961, and ligating this fragment to a fragment 1561 base pairs in length isolated after a partial digestion of pRCL6 with Sau 961 Ligation was performed in 50 mM trisHCl (pH7.4) 10 mM $MgCl_2$, 10 mM dithiothreitol. 1 mM spermidine, 10 mM ATP, 0.1 mg/ml BSA with 5 units commercial T4 DNA ligase, and incubation proceeded overnight at 15°C. After a standard phenol/chloroform extraction and ethanol precipitation the ligated DNA was pelleted, dissolved in a small volume of 10 mM tris HCl (pH 7.4), 1mM EDTA and digested to completion with Pst I. The resulting linearised DNA was then ligated (as above) with an equal quantity of Pst I linearised, phosphatased pUC8. The new recombinant DNA molecule containing the entire DNA sequence of the ricin precursor and referred to as pRCL617, was used in conventional manner to transform E. coli DHI cells.

The nucleotide sequence of pRCL617 is shown hereinafter.

This sequence was deduced from the two overlapping cDNA inserts in clones pRCL6 and pRCL17 (the limits of the DNA inserts in each of these two clones are given below).

Nucleotide residues are numbered in the 5' to 3' direction with the first residue of the codon specifying the amino terminal residue of mature ricin A chain numbered 1 and the nucleotides on the 5' side of residue 1 indicated by negative numbers. The 5' terminal sequence does not extend to the 5' end of the mRNA whereas the 3' terminal sequence shown is followed by a poly (dA) tract 27 residues long, thus representing the complete sequence of the region. The predicted amino acid sequence is given below the nucleotide sequence and differences with the published amino acid sequence of mature ricin A and B chains (Funatsu G., Kimura, M and Funatsu, M. Agric.Biol. Chem. Vol 43, pp 2221-2224 (1979), and Yoshitake, S., Funatsu, G and Funatsu, M - Agric. Biol. Chem. Vol.42, pp 1267-1274 (1978)) are indicated underneath. Residues absent from the published amino acid sequence are underlined with a dashed line and the position of amino acids present in the published sequence but absent from the derived sequence presented here are indicated by an asterisk. The dashed line beneath the

12 amino acid sequence linking the C-terminus of the A chain and the N-terminus of the B chain is bracketed. Amino acids are numbered from the amino terminal residue of the mature A chain and the preceding residues are indicated by negative numbers. Potential sites for asparagine linked N-glycosylation are boxed and potential poly (A) signals are underlined. The insert of pRCL6 extends from nucleotide - 102 to residue 1512 and the insert of pRCL17 extends from nucleotide 733 to residue 1782.

The intervening twelve triplets code for the linker amino acid sequence which is present in the precursor polypeptide and which is enzymatically removed in the cell to separate the A and B chains. which chains arejoined by a disulphide bridge during the formation of the ricin molecule itself. This linker region as well as the presumptive amino terminal leader or signal sequence (amino acids - 24 to -1) are not present in the sequences already published by Funatsu et al.

Preproricin is the whole polypeptide coded for by the aforesaid DNA insert, i.e. from amino acid -24 to amino acid 541. Proricin, which is obtained from preproricin in the organism by removal of the amino acid leader sequence, extends from amino acid 1 to amino acid 541.

0145111

```
-100                                                              -50
5'--AAACCGGGAG GAAATACTAT TGTAATATGG ATG TAT GCA GTG GCA ACA TGG CTT
                                     Met Tyr Ala Val Ala Thr Trp Leu
                                                     -20

                                                             -1  1
TGT TTT GGA TCC ACC TCA GGG TGG TCT TTC ACA TTA GAG GAT AAC AAC ATA
Cys Phe Gly Ser Thr Ser Gly Trp Ser Phe Thr Leu Glu Asp Asn Asn Ile
                        -10                                  -1  1

                                                             50
TTC CCC AAA CAA TAC CCA ATT ATA AAC TTT ACC ACA GCG GGT GCC ACT GTG
Phe Pro Lys Gln Tyr Pro Ile Ile [Asn Phe Thr] Thr Ala Gly Ala Thr Val
                                10

                                                             100
CAA AGC TAC ACA AAC TTT ATC AGA GCT GTT CGC GGT CGT TTA ACA ACT GGA
Gln Ser Tyr Thr Asn Phe Ile Arg Ala Val Arg Gly Arg Leu Thr Thr Gly
    20                                  30

                                                         150
GCT GAT GTG AGA CAT GAT ATA CCA GTG TTG CCA AAC AGA GTT GGT TTG CCT
Ala Asp Val Arg His Asp Ile Pro Val Leu Pro Asn Arg Val Gly Leu Pro
                40  Glu                                 50

                                                     200
ATA AAC CAA CGG TTT ATT TTA GTT GAA CTC TCA AAT CAT GCA GAG CTT TCT
Ile Asn Gln Arg Phe Ile Leu Val Glu Leu Ser Asn His Ala Glu Leu Ser
                            60          Gln

                                                 250
GTT ACA TTA GCC CTG GAT GTC ACC AAT GCA TAT GTG GTC GGC TAC CGT GCT
Val Thr Leu Ala Leu Asp Val Thr Asn Ala Tyr Val Val Gly Tyr Arg Ala
70                  Ser                 80

                                             300
GGA AAT AGC GCA TAT TTC TTT CAT CCT GAC AAT CAG GAA GAT GCA GAA GCA
Gly Asn Ser Ala Tyr Phe Phe His Pro Asp Asn Gln Glu Asp Ala Glu Ala
            90                                  100

                                             350
ATC ACT CAT CTT TTC ACT GAT GTT CAA AAT CGA TAT ACA TTC GCC TTT GGT
Ile Thr His Leu Phe Thr Asp Val Gln Asn Arg Tyr Thr Phe Ala Phe Gly
                        110                                  120
```

Page 30 a follows

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                                                                400
GGT AAT TAT GAT AGA CTT GAA CAA CTT GCT GGT AAT CTG AGA GAA AAT ATC
Gly Asn Tyr Asp Arg Leu Glu Gln Leu Ala Gly Asn Leu Arg Glu Asn Ile
                                    130

                                                            450
GAG TTG GGA AAT GGT CCA CTA GAG GAG GCT ATC TCA GCG CTT TAT TAT TAC
Glu Leu Gly Asn Gly Pro Leu Glu Glu Ala Ile Ser Ala Leu Tyr Tyr Tyr
        140                                                 150

                                                        500
AGT ACT GGT GGC ACT CAG CTT CCA ACT CTG GCT CGT TCC TTT ATA ATT TGC
Ser Thr Gly Gly Thr Gln Leu Pro Thr Leu Ala Arg Ser Phe Ile Ile Cys
                    160                                         170

                                                        550
ATC CAA ATG ATT TCA GAA GCA GCA AGA TTC CAA TAT ATT GAG GGA GAA ATG
Ile Gln Met Ile Ser Glu Ala Ala Arg Phe Gln Tyr Ile Glu Gly Glu Met
                                180

                                                    600
CGC ACG AGA ATT AGG TAC AAC CGG AGA TCT GCA CCA GAT CCT AGC GTA ATT
Arg Thr Arg Ile Arg Tyr Asn Arg Arg Ser Ala Pro Asp Pro Ser Val Ile
    190                                             200

                                                    650
ACA CTT GAG AAT AGT TGG GGG AGA CTT TCC ACT GCA ATT CAA GAG TCT AAC
Thr Leu Glu Asn Ser Trp Gly Arg Leu Ser Thr Ala Ile Gln Glu Ser Asn
                210                                         220

                                                    700
CAA GGA GCC TTT GCT AGT CCA ATT CAA CTG CAA AGA CGT AAT GGT TCC AAA
Gln Gly Ala Phe Ala Ser Pro Ile Gln Leu Gln Arg Arg[Asn Gly Ser]Lys
                            230                     --- Asp

                                                750
TTC AGT GTG TAC GAT GTG AGT ATA TTA ATC CCT ATC ATA GCT CTC ATG GTG
Phe Ser Val Tyr Asp Val Ser Ile Leu Ile Pro Ile Ile Ala Leu Met Val
240                                     Leu 250             ---

                                                800
TAT AGA TGC GCA CCT CCA CCA TCG TCA CAG TTT TCT TTG CTT ATA AGG CCA
Tyr Arg Cys Ala Pro Pro Pro Ser Ser Gln Phe Ser Leu Leu Ile Arg Pro
            260                                 (--- --- --- --- --- ---
```

Page 30b follows

```
                                                    850
GTG GTA CCA AAT TTT AAT GCT GAT GTT TGT ATG GAT CCT GAG CCC ATA GTG
Val Val Pro Asn Phe Asn Ala Asp Val Cys Met Asp Pro Glu Pro Ile Val
--- --- --- --- --- ---)280                              290

                                                900
CGT ATC GTA GGT CGA AAT GGT CTA TGT GTT GAT GTT AGG GAT GGA AGA TTC
Arg Ile Val Gly Arg Asn Gly Leu Cys Val Asp Val Arg Asp Gly Arg Phe
                                    300 Asn

                                                950
CAC AAC GGA AAC GCA ATA CAG TTG TGG CCA TGC AAG TCT AAT ACA GAT GCA
His Asn Gly Asn Ala Ile Gln Leu Trp Pro Cys Lys Ser Asn Thr Asp Ala
Asn His 310                                     320

                                               1000
AAT CAG CTC TGG ACT TTG AAA AGA GAC AAT ACT ATT CGA TCT AAT GGA AAG
Asn Gln Leu Trp Thr Leu Lys Arg Asp Asn Thr Ile Arg Ser Asn Gly Lys
          ---     330                                       340

                                          1050
TGT TTA ACT ACT TAC GGG TAC AGT CCG GGA GTC TAT GTG ATG ATC TAT GAT
Cys Leu Thr Thr Tyr Gly Tyr Ser Pro Gly Val Tyr Val Met Ile Tyr Asp
                               Pro Ser

                                     1100
TGC AAT ACT GCT GCA ACT GAT GCC ACC CGC TGG CAA ATA TGG GAT
Cys Asn Thr Ala Ala Thr Asp Ala Thr Arg Trp Gln Ile Trp Asp
    360                 Thr     Asp     --- Glu         Asn

                                               1150
AAT GGA ACC ATC ATA AAT CCC AGA TCT AGT CTA GTT TTA GCA GCG ACA TCA
Asn Gly Thr Ile Ile Asn Pro Arg Ser Ser Leu Val Leu Ala Ala Thr Ser
                        380                                     390

                                               1200
GGG AAC AGT GGT ACC ACA CTT ACG GTG CAA ACC AAC ATT TAT GCC GTT AGT
Gly Asn Ser Gly Thr Thr Leu Thr Val Gln Thr Asn Ile Tyr Ala Val Ser
                                    400

                                               1250
CAA GGT TGG CTT CCT ACT AAT AAT ACA CAA CCT TTT GTT ACA ACC ATT GTT
Gln Gly Trp Leu Pro Thr Asn Asn Thr Gln Pro Phe Val Thr Thr Ile Val
        Pro     Phe                             Trp 420
```

Page 30c follows...

```
                                    1300
GGG CTA TAT GGT CTG TGC TTG CAA GCA AAT AGT GGA CAA GTA TGG ATA GAG
Gly Leu Tyr Gly Leu Cys Leu Gln Ala Asn Ser Gly Gln Val Trp Ile Glu
                    430                                     Val 440

                                1350
GAC TGT AGC AGT GAA AAG GCT GAA CAA CAG TGG GCT CTT TAT GCA GAT GGT
Asp Cys Ser Ser Glu Lys Ala Glu Gln Gln Trp Ala Leu Tyr Ala Asp Gly
    Ser Cys                     450                             Ser

                            1400
TCA ATA CGT CCT CAG CAA AAC CGA GAT AAT TGC CTT ACA AGT GAT TCT AAT
Ser Ile Arg Pro Gln Gln Asn Arg Asp Asn Cys Leu Thr Ser Asp Ser Asn
Asn 460 Asn             Arg                 470

                            1450
ATA CGG GAA ACA GTT GTT AAG ATC CTC TCT TGT GGC CCT GCA TCC TCT GGC
Ile Arg Glu Thr Val Val Lys Ile Leu Ser Cys Gly Pro Ala Ser Ser Gly
                480                                     490

                            1500
CAA CGA TGG ATG TTC AAG AAT GAT GGA ACC ATT TTA AAT TTG TAT AGT GGA
Gln Arg Trp Met Phe Lys Asn Asp Gly Thr Ile Leu Asn Leu Tyr Ser Gly
Glu                         500

                            1550
TTG GTG TTA GAT GTG AGG CGA TCG GAT CCG AGC CTT AAA CAA ATC ATT CTT
Leu Val Leu Asp Val Arg Arg Ser Asp Pro Ser Leu Lys Gln Ile Ile Leu
 510                    Ala             520

                            1600
TAC CCT CTC CAT GGT GAC CCA AAC CAA ATA TGG TTA CCA TTA TTT TGA
Tyr Pro Leu His Gly Asp Pro Asn Gln Ile Trp Leu Pro Leu Phe ***
            Trp    *                --- ---    *Leu Pro

                        1650
TAGACAGATT ACTCTCTTGC AGTGTGTGTG TCCTGCCATG AAAATAGATG GCTTAAATAA

            1700
AAAGGACATT GTAAATTTTG TAACTGAAAG GACAGCAAGT TATTGCAGTC CAGTATCTAA

1750                                1780
TAAGAGCACA ACTATTGTCT TGTGCATTCT AAATTT-Poly(A)
```

CLAIMS:

1. DNA comprising a nucleotide sequence coding for at least a substantial portion of a plant toxin of the ricin-type or a mutant thereof, characterised in that it is biologically pure and homogeneous.

2. DNA according to claim 1, characterised in that the nucleotide sequence codes for the A chain or the B chain of the mature toxin.

3. DNA according to claim 1, 2 or 3, characterised in that the nucleotide sequence codes for a mutant in which the galactose binding site or sites have been removed or inactivated.

4. A sample of DNA including at least a substantial portion of the following nucleotide sequence:

ATG TAT GCA GTG GCA ACA TGG CTT

TGT TTT GGA TCC ACC TCA GGG TGG TCT TTC ACA TTA GAG GAT AAC AAC ATA

TTC CCC AAA CAA TAC CCA ATT ATA AAC TTT ACC ACA GCG GGT GCC ACT GTG

CAA AGC TAC ACA AAC TTT ATC AGA GCT GTT CGC GGT CGT TTA ACA ACT GGA

GCT GAT GTG AGA CAT GAT ATA CCA GTG TTG CCA AAC AGA GTT GGT TTG CCT

ATA AAC CAA CGG TTT ATT TTA GTT GAA CTC TCA AAT CAT GCA GAG CTT TCT

GTT ACA TTA GCC CTG GAT GTC ACC AAT GCA TAT GTG GTC GGC TAC CGT GCT

GGA AAT AGC GCA TAT TTC TTT CAT CCT GAC AAT CAG GAA GAT GCA GAA GCA

ATC ACT CAT CTT TTC ACT GAT GTT CAA AAT CGA TAT ACA TTC GCC TTT GGT

Page 32a follows

GGI AAT TAT GAT AGA CTT GAA CAA CTI GCI GGI AAT CTG AGA GAA AAT ATC

GAG TTG GGA AAT GGT CCA CTA GAG GAG GCT ATC TCA GCG CTT TAT TAT TAC

AGT ACT GGI GGC ACT CAG CTT CCA ACT CTG GCT CGI TCC TTT ATA ATT TGC

ATC CAA ATG ATT TCA GAA GCA GCA AGA TTC CAA TAT ATT GAG GGA GAA ATG

5. CGC ACG AGA ATT AGG TAC AAC CGG AGA TCT GCA CCA GAT CCT AGC GTA ATT

ACA CTT GAG AAT AGT TGG GGG AGA CTT TCC ACT GCA ATT CAA GAG TCT AAC

CAA GGA GCC TTT GCT AGT CCA ATT CAA CTG CAA AGA CGT AAT GGT TCC AAA

TTC AGT GTG TAC GAT GTG AGT ATA TTA ATC CCT ATC ATA GCT CTC ATG GTG

TAT AGA TGC GCA CCT CCA CCA TCG TCA CAG TTT TCT TTG CTT ATA AGG CCA

Page 32b follows...

GTG GTA CCA AAT TTT AAT GCT GAT GTT TGT ATG GAT CCT GAC CCC ATA GTG

CGT ATC GTA GGT CGA AAT GGT CTA TGT GTT GAT GTT AGG GAT GGA AGA TTC

CAC AAC GGA AAC GCA ATA CAG TTG TGG CCA TGC AAG TCT AAT ACA GAT GCA

AAT CAG CTC TGG ACT TTG AAA AGA GAC AAT ACT ATT CGA TCT AAT GGA AAG

TGT TTA ACT ACT TAC GGG TAC AGT CCG GGA GTC TAT GTG ATG ATC TAT GAT

TGC AAT ACT GCT GCA ACT GAT GCC ACC CGC TGG CAA ATA TGG GAT

AAT GGA ACC ATC ATA AAT CCC AGA TCT AGT CTA GTT TTA GCA GCG ACA TCA

GGG AAC AGT GGT ACC ACA CTT ACG GTG CAA ACC AAC ATT TAT GCC GTT AGT

CAA GGT TGG CTT CCT ACT AAT AAT ACA CAA CCT TTT GTT ACA ACC ATT GTT

Page 32c follows...

GGG CTA TAT GGT CTG TGC TTG CAA GCA AAT AGT GGA CAA GTA TGG ATA GAG

GAC TGT AGC AGT GAA AAG GCT GAA CAA CAG TGG GCT CTT TAT GCA GAT GGT

TCA ATA CGT CCT CAG CAA AAC CGA GAT AAT TGC CTT ACA AGT GAT TCT AAT

ATA CGG GAA ACA GTT GTT AAG ATC CTC TCT TGT GGC CCT GCA TCC TCT GGC

CAA CGA TGG ATG TTC AAG AAT GAT GGA ACC ATT TTA AAT TTG TAT AGT GGA

TTG GTG TTA GAT GTG AGG CGA TCG GAT CCG AGC CTT AAA CAA ATC ATT CTT

TAC CCT CTC CAT GGT GAC CCA AAC CAA ATA TGG TTA CCA TTA TTT

or at least a portion of a nucleotide sequence that is equivalent thereto by virtue of degeneracy of the genetic code.

5. A sample of DNA including at least a substantial portion of the following nucleotide sequence:

ATG TAT GCA GTG GCA ACA TGG CTT

TGT TTT GGA TCC ACC TCA GGG TGG TCT TTC ACA TTA GAG GAT AAC AAC ATA

TTC CCC AAA CAA TAC CCA ATT ATA AAC TTT ACC ACA GCG GGT GCC ACT GTG

CAA AGC TAC ACA AAC TTT ATC AGA GCT GTT CGC GGT CGT TTA ACA ACT GGA

GCT GAT GTG AGA CAT GAT ATA CCA GTG TTG CCA AAC AGA GTT GGT TTG CCT

ATA AAC CAA CGG TTT ATT TTA GTT GAA CTC TCA AAT CAT GCA GAG CTT TCT

GTT ACA TTA GCC CTG GAT GTC ACC AAT GCA TAT GTG GTC GGC TAC CGT GCT

GGA AAT AGC GCA TAT TTC TTT CAT CCT GAC AAT CAG GAA GAT GCA GAA GCA

ATC ACT CAT CTT TTC ACT GAT GTT CAA AAT CGA TAT ACA TTC GCC TTT GGT

Page 33a follows...

GGI AAI TAT GAT AGA CTT GAA CAA CTI GCI GGI AAT CTG AGA GAA AAT AIC

GAG TTG GGA AAT GGT CCA CTA GAG GAG GCI ATC TCA GCG CTT TAT TAT TAC

AGT ACT GGT GGC ACT CAG CTT CCA ACT CTG GCI CGI TCC TTI ATA ATT TGC

ATC CAA ATG ATT TCA GAA GCA GCA AGA TTC CAA TAT ATT GAG GGA GAA ATG

CGC ACG AGA ATI AGG TAC AAC CGA AGA TCI GCA CCA GAT CCI AGC GTA ATI

ACA CTI GAG AAT AGT TGG GGG AGA CTT TCC ACT GCA ATT CAA GAG TCI AAC

CAA GGA GCC TTI GCI AGT CCA ATI CAA CTG CAA AGA CGI AAT GGI TCC AAA

TTC AGI GTG TAC GAT GTG AGI ATA TTA AIC CCI AIC ATA GCI CTC ATG GTG

TAT AGA TGC GCA CCI CCA CCA TCG TCA CAG TTI ,

or at least a portion of a nucleotide sequence which is equivalent thereto by virtue of degeneracy of the genetic code.

6. A sample of DNA including at least a substantial portion of the following nucleotide sequence:

GCT GAT GTT TGT ATG GAT CCT GAG CCC ATA GTG

CGT ATC GTA GGT CGA AAT GGT CTA TGT GTT GAT GTT AGG GAT GGA AGA TTC

CAC AAC GGA AAC GCA ATA CAG TTG TGG CCA TGC AAG TCT AAT ACA GAT GCA

AAT CAG CTC TGG ACT TTG AAA AGA GAC AAT ACT ATT CGA TCT AAT GGA AAG

TGT TTA ACT ACT TAC GGG TAC AGT CCG GGA GTC TAT GTG ATG ATC TAT GAT

TGC AAT ACT GCT GCA ACT GAT GCC ACC CGC TGG CAA ATA TGG GAT

AAT GGA ACC ATC ATA AAT CCC AGA TCT AGT CTA GTT TTA GCA GCG ACA TCA

GGG AAC AGT GGT ACC ACA CTT ACG GTG CAA ACC AAC ATT TAT GCC GTT AGT

CAA GGT TGG CTT CCT ACT AAT AAT ACA CAA CCT TTT GTT ACA ACC ATT GTT

Page 34a follows...

GGG CTA TAT GGT CTG TGC TTG CAA GCA AAT AGT GGA CAA GTA TGG ATA GAG

GAC TGT AGC AGT GAA AAG GCT GAA CAA CAG TGG GCT CTT TAT GCA GAT GGT

TCA ATA CGT CCT CAG CAA AAC CGA GAT AAT TGC CTT ACA AGT GAT TCT AAT

ATA CGG GAA ACA GTT GTT AAG ATC CTC TCT TGT GGC CCT GCA TCC TCT GGC

CAA CGA TGG ATG TTC AAG AAT GAT GGA ACC ATT TTA AAT TTG TAT AGT GGA

TTG GTG TTA GAT GTG AGG CGA TCG GAT CCG AGC CTT AAA CAA ATC ATT CTT

TAC CCT CTC CAT GGT GAC CCA AAC CAA ATA TGG TTA CCA TTA TTT

or at least a portion of a nucleotide sequence which is equivalent thereto by virtue of degeneracy of the genetic code.

7. A recombinant DNA molecule, characterised in that it includes as an insert a nucleotide sequence as defined in any preceding claim.

8. A recombinant DNA molecule according to claim 7, characterised in that said insert has been introduced

into a cl ning vector which is a plasmid or a bacteriophage.

9. A modified host cell containing a recombinant DNA molecule according to claim 7 or claim 8.

10. A modified host cell according to claim 9, characterised in that the host is a plant cell, an animal cell, a Gram-negative bacterium, a Gram-positive bacterium, or a yeast.

11. A modified host microorganism according to claim 10, characterised in that the host is a Gram-negative bacterium, which is any of E.coli, Methylophilus methylotropus, or Alcaligenes eutrophus.

12. A modified host microorganism according to claim 10, characterised in that the host is a Gram-positive bacterium which is any of Streptomyces, Bacillus or Arthrobacter.

13. A modified host microorganism according to claim 10, characterised in that the host is a yeast which is Saccharomyces cervisiae.

14. A modified host microorganism according to

claim 11, wherein the cloning vector is any suitable plasmid sel ct d from pBR322, pAT153, pUC8, pGS15 or pMB9.

15. A modified host microorganism according to claim 12, wherein the cloning vector is the plasmid pUC6 .

16. A modified host microorganism according to claim 13, when the cloning vector is pMA91, pMA230, YRp7, pLC544, and YEp6.

17. Method of preparing a biologically-pure sample of a cDNA sequence coding for a precursor ,or a portion thereof, of a plant toxin of the ricin type, characterised by isolating mRNA from the tissue of a plant which produces such a toxin, and synthesising cDNA from this by reverse transcription.

18. A method of obtaining a recombinant DNA molecule by inserting a double-stranded cDNA according to claim 17 into a cloning vector.

19. A method of obtaining a genetically-modified host characterised in that a recombinant DNA molecule according to claim 18 is introduced into a host micro-organism.

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6th November 1984

Dear Sirs,

European Patent Application No. 84304801.8
The University of Warwick

In response to the Official Communication (EPO Form 1150) of the 29th August 1984, we now enclose in triplicate re-typed pages 7, 7a, 7b, 7c, 8, 8a, 8b, 8c, 30, 30a, 30b, 32, 32a, 32b, 32c, 33 and 34.

The four codons TTA CCA TTA TTT have been added to the end of claim 4, to bring the claim into exact agreement with the statement on original page 7. We submit that it would be obvious to one skilled in the art that the DNA sequence of claim 4 should be identical to the sequence on original page 7, and also to those portions of the DNA sequence given on original page 30 which are expressed as polypeptides and that this amendment is therefore allowable under Rule 88.

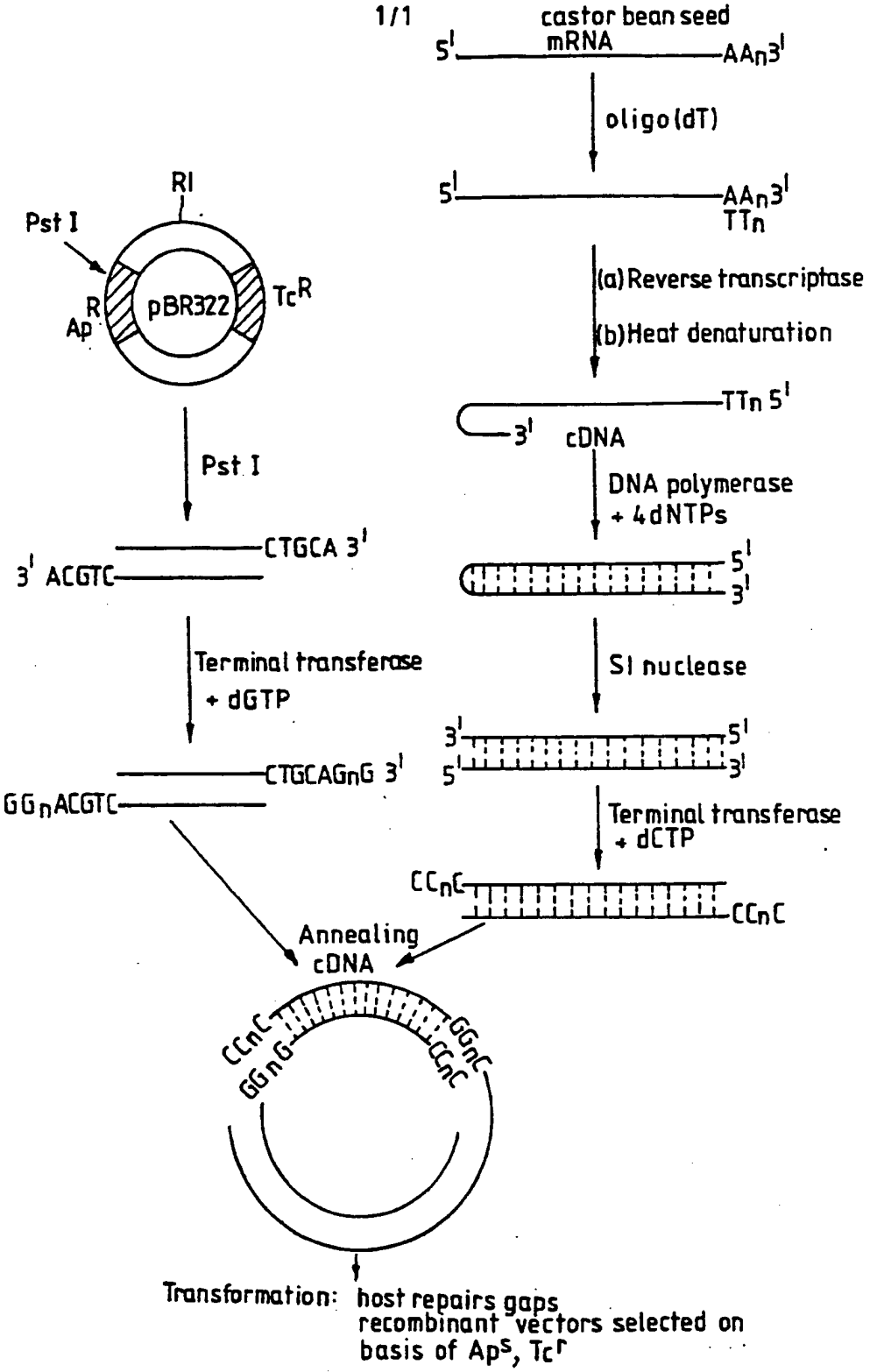
We shall be grateful if you will acknowledge receipt of these documents using the enclosed copy letter and self-addressed envelope.

Yours faithfully,

D. G. Bannerman
WITHERS & ROGERS

The request for correction is allowed under R. 88 EPC / ~~with the exception of the deleted points~~.
THE HAGUE G. SENELLE 04 FEB. 1985
RECEIVING SECTION

Encls.


castor bean seed
mRNA
5'
AAn3'
oligo(dT)
AAn3'
TTn
(a) Reverse transcriptase
(b) Heat denaturation
TTn 5'
3'
cDNA
DNA polymerase
+ 4dNTPs
5'
3'
S1 nuclease
3'
5'
5'
3'
Terminal transferase
+ dCTP
CCnC
CCnC
RI
Pst I
pBR322
TcR
ApR
Pst I
CTGCA 3'
3' ACGTC
Terminal transferase
+ dGTP
CTGCAGnG 3'
GGnACGTC
Annealing
cDNA
CCnC
GGnG
GGnC
CCnC
Transformation:
host repairs gaps
recombinant vectors selected on
basis of Aps, Tcr

European Patent Office

EUROPEAN SEARCH REPORT

0145111
Application number

DOCUMENTS CONSIDERED TO BE RELEVANT

EP 84304801.8

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl 4)
A,D	AGRICULTURAL AND BIOLOGICAL CHEMISTRY, vol. 42, no. 5, May 1978, Tokyo S. YOSHITAKE et al. "Isolation and Sequences of Peptic Peptides, and the Complete Sequence of Ile Chain of Ricin D" pages 1267-1274 * Pages 1270-1272 * --	1,2	C 12 N 15/00 C 12 P 21/00 //C 12 R 1:19 C 12 R 1:05 C 12 R 1:465 C 12 R 1:07 C 12 R 1:06 C 12 R 1:865
A,D	AGRICULTURAL AND BIOLOGICAL CHEMISTRY, vol. 43, no. 9, September 1979, Tokyo G. FUNATSU et al. "Primary Structure of Ala Chain of Ricin D" pages 2221-2224 * Totality * ----	1,2	

TECHNICAL FIELDS SEARCHED (Int. Cl 4)

C 12 N
C 12 P

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	19-10-1984	WOLF

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
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P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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(54) **DNA sequence coding for a plant toxin of the ricin type, or a portion thereof.**

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(56) References cited:

- **Wo 85103508**
- **Eur.J. Biochem. 119, 31-41 (1981)**
- **Eur.J. Biochem. 137, 57-65 (1983)**

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EP 0 145 111 B1

Courier Press, Leamington Spa, England.

Description

This invention relates to DNA comprising a nucleotide sequence coding for at least a portion of a polypeptide which is a plant t xin of the ricin type, as hereinafter defined. It also relates to recombinant DNA molecules c mprising a DNA sequence which codes for a polypeptide which is r is closely related to a natural plant toxin of the ricin type. Ricin, and also other plant toxins such as abrin, modeccin and viscumin, consist of two polypeptide chains (known as the A and B chains) linked by a disulphide bridge, one chain (the A chain) being primarily responsible for the cytotoxic and the other chain (the B chain) having sites enabling the molecule to bind to cell surfaces. Ricin is produced in the plant *Ricinis communis* (also known as the castor bean plant) via a precursor protein known as "preproricin".

Preproricin comprises a single polypeptide chain which includes a leader sequence. The leader sequence is subsequently removed in the organism to give proricin which is then cleaved to eliminate a linker region and joined by a disulphide bond to form the mature protein.

The toxicity of ricin-type toxins operates in three phases: (1) binding to the cell surface via the B chain; (2) penetration of at least the A chain into the cytosol, and (3) inhibition of protein synthesis through the A chain attacking the 60S subunits of the ribosomes. Thus, separated A and B chains are essentially non-toxic, the inherently toxic A chain lacking the ability to bind to cell surfaces in the absence of the B chain.

It is also known that in ricin-type toxins the B chain binds to cell surfaces by virtue of galactose recognition sites, which react with glycoproteins or glycolipids exposed at the cell surface.

It has already been suggested that the toxicity of the ricin A chain might be exploited in anti-tumour therapy, by replacing the indiscriminately-binding B chain with a different carrier component having the ability to bind only to tumour cells. Thus, various immunotoxins have already been prepared, consisting of a conjugate of whole ricin or a separated natural ricin A chain and a tumour-specific monoclonal antibody. Although these known conjugates are of considerable potential in themselves, there is scope for improvement.

One problem with the known conjugates arises from a structural feature of the A chain from natural ricin. It is known that the natural ricin A chain becomes N-glycosylated during its synthesis, by enzymes present in *Ricinus* cells, and it is thought that the resulting sugar moieties are capable of non-specific interactions with cell surfaces. Thus, it appears that the known A chain conjugates are capable of a certain amount of binding with non target cells, even in the absence of the natural B chain, thus increasing the toxicity of such immunotoxins towards non target cells.

Another problem with the known ricin A chain conjugates stems from the fact that the B chain seems to have an important secondary function in that it somehow assists in the intoxication process, apart from its primary function in binding the ricin molecule to the cell surface. This secondary function is lost if the B chain is replaced by a different carrier component such as a monoclonal antibody.

If it were possible to prevent interactions between the cell surface via the A chain sugar moieties, whilst preserving the secondary toxicity-increasing function of the B chain, the toxicity of a whole ricin antibody conjugate towards normal cells could be reduced, and towards target cells could be increased, thus improving the therapeutic index of the immunotoxin. It is also known that the natural ricin B chain is N-glycosylated and the B chain sugar moieties may also contribute to non specific interactions. Also, the sugar moieties in both chains enable the ricin molecule to be sequestrated by reticuloendothelial cells in the liver, and so would lead to the rapid excretion from the system of a drug based on a part or the whole of the ricin molecule in which such sugar moieties were still present.

Attempts to remove all the sugar moieties from natural ricin by chemical or enzymatic methods have so far failed. Nevertheless the major obstacle confronting the use of known whole ricin-antibody conjugates is the presence of two galactose binding sites in the ricin B chain. These B chain galactose binding sites are primarily responsible for the non-specific cellular interactions of current whole ricin-antibody conjugates, particularly when used *in vivo*. Their presence in the natural toxin clearly eliminates or reduces the targeting specifity conferred by the antibody.

An improved immunotoxin based on ricin or another plant toxin of the ricin type, not suffering from these problems, could consist of a whole toxin molecule modified so that it is not N-glycosylated, and so that the B chain has no galactose recognition sites, but retains its secondary intoxication-promoting properties, coupled to a carrier moeity which delivers the toxin to the target cells. This could be a tumour-specific or cell/tissue specific vehicle such as a suitable monoclonal antibody.

Our research which has so far been concentrated on ricin itself, has indicated that the assembly of ricin (and the related agglutinin which consists of two ricin-like molecules with slightly modified A and B chains) does not involve the separate synthesis of the A and B chains as the products of distinct mRNA's, but rather the initial formation of a single polypeptide precursor containing both the A chain and B chain sequences. This is thought to apply in the case of other toxins of the same type.

This invention is based on the idea of preparing a genetically-engineered microorganism capable of xpressing a molecule of a toxin of the ricin typ , as defined above, or alternatively part of such a toxin molecule, or a precurs r of such a molecule (which c uld be convert d to the toxin molecul itself) which toxin molecule could be modified as suggested above and could be used to construct an effective toxin conjugate by combining it with a tumour-specific or cell/tissue specific monocl nal antibody or ther carrier moiety, such as a hormone or lectin.

The fact that ricin is formed via a precursor polypeptide will enable a cell system to be constructed by known techniques which expresses a ricin precursor. The ricin precursor product could then be chemically or enzymatically converted to the d sired modified ricin. An analag us technique could be used in the case of other ricin-type toxins as h r in defined. An alternative technique would be t divide from the DNA sequence that c des for the precursor tw sequences which code separately fr m the A and B chains, to insert these separated sequences into different cloning vehicles and to insert the resulting recombinant DNA molecules into separate host microorganisms. One such host would then express the A chain polypeptide sequence and the other the B chain polypeptide sequence. These sequences could then be combined to form the desired modified ricin molecule. This technique could obviously also be used for any ricin-type toxins in which the A and B chains are encoded by distinct mRNA gene pools. This latter approach would be preferred on safety grounds, in that separate and therefore non-toxic A and B chains would be expressed.

According to one aspect of the invention we provide a biologically pure and homogeneous sample of DNA comprising a nucleotide sequence coding for at least a portion of a precursor of a ricin-type toxin polypeptide, or mutants thereof said portion coding for a polypeptide that displays the ribosome-inactivity property of the A chain of ricin.

Said portion preferably comprises the A chain of the mature protein.

More specifically, we provide a sample of DNA including at least a substantial portion of any of the following DNA sequences, said portion coding for a polypeptide that displays the ribosome-inactivity property of the A chain of ricin, which sample is biologically-pure:

ATG TAT GCA GTG GCA ACA TGG CTT

TGT TIT GGA TCC ACC TCA GGG TGG TCT TTC ACA TTA GAG GAT AAC AAC ATA

TTC CCC AAA CAA TAC CCA ATT ATA AAC TTT ACC ACA GCG GGI GCC ACT GTG

CAA AGC TAC ACA AAC TTT ATC AGA GCT GTT CGC GGT CGT TIA ACA ACT GGA

GCT GAT GTG AGA CAT GAT ATA CCA GTG TIG CCA AAC AGA GTT GGT TTG CCT

ATA AAC CAA CGG TTT ATT TTA GTT GAA CTC TCA AAT CAT GCA GAG CTT TCT

GTT ACA TTA GCC CTG GAT GTC ACC AAT GCA TAT GTG GTC GGC TAC CGT GCT

GGA AAT AGC GCA TAT TTC TTT CAT CCT GAC AAT CAG GAA GAT GCA GAA GCA

ATC ACT CAT CTT TTC ACT GAT GTT CAA AAT CGA TAT ACA TTC GCC TTT GGT

GGT AAT TAT GAT AGA CTT GAA CAA CTT GCT GGT AAT CTG AGA GAA AAT ATC

GAG TTG GGA AAT GGT CCA CTA GAG GAG GCT ATC TCA GCG CTT TAT TAT TAC

AGT ACT GGT GGC ACT CAG CTT CCA ACT CTG GCT CGT TCC TTT ATA ATT TGC

ATC CAA ATG ATT TCA GAA GCA GCA AGA TTC CAA TAT ATT GAG GGA GAA ATG

CGC ACG AGA ATT AGG TAC AAC CGG AGA TCT GCA CCA GAT CCT AGC GTA ATT

ACA CTT GAG AAT AGT TGG GGG AGA CTT TCC ACT GCA ATT CAA GAG TCT AAC

CAA ĠGA GCC TTT GĊT AGT CCA ATṪ CAA CTG CAA ȦGA CGT AAT GĠT TCC AAA

TTĊ AGT GTG TAC ĠAT GTG AGT ATȦ TTA ATC CCṪ ATC ATA GCT ĊTC ATG GTG

TȦT AGA TGC GCȦ CCT CCA CCA ṪCG TCA CAG TṪT TCT TTG CTṪ ATA AGG CCA

ĠTG GTA CCA AȦT TTT AAT GCṪ GAT GTT TGT ȦTG GAT CCT GȦG CCC ATA GTĠ

CGT ATC GTA GGT CGA AAT GĠT CTA TGT GTṪ GAT GTT AGG ĠAT GGA AGA TṪC

CAC AAC GGȦ AAC GCA ATA ĊAG TTG TGG CĊA TGC AAG TCṪ AAT ACA GAT ĠCA

AAT CAG CṪC TGG ACT TTĠ AAA AGA GAC ȦAT ACT ATT CĠA TCT AAT GGȦ AAG

TGT TTA ȦCT ACT TAC GĠG TAC AGT CCĠ GGA GTC TAT GTG ATG ATC TȦT GAT

TGC AAṪ ACT GCT GCA ȦCT GAT GCC AĊC CGC TGG CAȦ ATA TGG GAT

ȦAT GGA ACC ATĊ ATA AAT CCĊ AGA TCT AGT ĊTA GTT TTA GĊA GCG ACA TCȦ

GGG AAC AGT ĠGT ACC ACA CṪT ACG GTG CAȦ ACC AAC ATT ṪAT GCC GTT AĠT

CAA GGT TGĠ CIT CCT ACT ȦAT AAT ACA CȦA CCT TTT GTṪ ACA ACC ATT ĠTT

GGG CTA TȦT GGT CTG TGĊ TTG CAA GCA ȦAT AGT GGA CȦA GTA TGG ATȦ GAG

GAC TGT ȦGC AGT GAA AȦG GCT GAA CAȦ CAG TGG GCT ĊTT TAT GCA GȦT GGT

TCA ATȦ CGT CCT CAG ĊAA AAC CGA GAT AAT TGC CTṪ ACA AGT GAT ṪCT AAT

ATA ĊGG GAA ACA GTṪ GTT AAG ATC ĊTC TCT TGT GĠC CCT GCA TCĊ TCT GGC

CAA ĊGA TGG ATG TṪC AAG AAT GAT GGA ACC ATT ṪTA AAT TTG TȦT AGT GGA

TTĠ GTG TTA GAT ĠTG AGG CGA TĊG GAT CCG AGĊ CTT AAA CAA ȦTC ATT CTT

TȦC CCT CTC CAṪ GGT GAC CCA AAC CAA ATA TĠG TTA CCA TTȦ TTT

ATĠ TAT GCA GTG ĠCA ACA TGG CṪT

TGT TIT GGȦ TCC ACC TCA ĠGG TGG TCT TṪC ACA TTA GAĠ GAT AAC AAĊ ȦTA

TTC CCC ȦAA CAA TAC CĊA ATT ATA AAĊ TTT ACC ACA GCG GGI GCC AĊT GTG

CAA AGC TAC ACA AAC TTT ATC AGA GCT GTT CGC GGT CGT TIA ACA ACT GGA

GCT GAT GTG AGA CAT GAT ATA CCA GTG TIG CCA AAC AGA GTT GGT TTG CCT

ATA AAC CAA CGG TTT ATT TTA GTT GAA CTC TCA AAT CAT GCA GAG CTT TCT

GTT ACA TTA GCC CTG GAT GTC ACC AAT GCA TAT GTG GTC GGC TAC CGT GCT

GGA AAT AGC GCA TAT TTC TTT CAT CCT GAC AAT CAG GAA GAT GCA GAA GCA

ATC ACT CAT CTT TTC ACT GAT GTT CAA AAT CGA TAT ACA TTC GCC TTT GGT

GGT AAT TAT GAT AGA CTT GAA CAA CTT GCT GGT AAT CTG AGA GAA AAT ATC

GAG TTG GGA AAT GGT CCA CTA GAG GAG GCT ATC TCA GCG CTT TAT TAT TAC

AGT ACT GGT GGC ACT CAG CTT CCA ACT CTG GCT CGT TCC TTT ATA ATT TGC

ATC CAA ATG ATT TCA GAA GCA GCA AGA TTC CAA TAT ATT GAG GGA GAA ATG

CGC ACG AGA ATT AGG TAC AAC CGG AGA TCT GCA CCA GAT CCT AGC GTA ATT

ACA CTT GAG AAT AGT TGG GGG AGA CTT TCC ACT GCA ATT CAA GAG TCT AAC

CAA GGA GCC TTT GCT AGT CCA ATT CAA CTG CAA AGA CGT AAT GGT TCC AAA

TTC AGT GTG TAC GAT GTG AGT ATA TTA ATC CCT ATC ATA GCT CTC ATG GTG

TAT AGA TGC GCA CCT CCA CCA TCG TCA CAG TTT

GCT GAT GTT TGT ATG GAT CCT GAG CCC ATA GTG

CGT ATC GTA GGT CGA AAT GGT CTA TGT GTT GAT GTT AGG GAT GGA AGA TTC

CAC AAC GGA AAC GCA ATA CAG TTG TGG CCA TGC AAG TCT AAT ACA GAT GCA

AAT CAG CTC TGG ACT TTG AAA AGA GAC AAT ACT ATT CGA TCT AAT GGA AAG

TGT TTA ACT ACT TAC GGG TAC AGT CCG GGA GTC TAT GTG ATG ATC TAT GAT

TGC AAT ACT GCT GCA ACT GAT GCC ACC CGC TGG CAA ATA TGG GAT

AAT GGA ACC ATC ATA AAT CCC AGA TCT AGT CTA GTT TTA GCA GCG ACA TCA

GGG AAC AGT ĠGT ACC ACA CṪT ACG GTG CAȦ ACC AAC ATT ṪAT GCC GTT AĠT

CAA GGT TGĠ CIT CCT ACT ȦAT AAT ACA CȦA CCT TTT GTṪ ACA ACC ATT ĠTT

GGG CTA TȦT GGT CTG TGĊ TTG CAA GCA ȦAT AGT GGA CȦA GTA TGG ATȦ GAG

GAC TGT ȦGC AGT GAA AȦG GCT GAA CAȦ CAG TGG GCT ĊTT TAT GCA GȦT GGT

TCA ATȦ CGT CCT CAG ĊAA AAC CGA GAT AAT TGC CTṪ ACA AGT GAT ṪCT AAT

ATA ĊGG GAA ACA GṪT GTT AAG ATC ĊTC TCT TGT GĠC CCT GCA TCĊ TCT GGC

CAA ĊGA TGG ATG TṪC AAG AAT GAT GGA ACC ATT ṪTA AAT TTG TȦT AGT GGA

TTĠ GTG TTA GAT ĠTG AGG CGA TĊG GAT CCG AGĊ CTT AAA CAA ȦTC ATT CTT

TȦC CCT CTC CAṪ GGT GAC CCA AAC CAA ATA TĠG TTA CCA TTȦ TTT

or at least a portion of a nucleotide sequence which is equivalent thereto by virtue of degeneracy of the genetic code.

According to another aspect of this invention we provide a recombinant DNA molecule comprising a DNA sequence which codes for a polypeptide sequence present in a plant toxin of the ricin type, as defined herein.

More specifically, we provide a recombinant DNA molecule containing a DNA sequence which codes for a A and B chain precursor polypeptide of a plant toxin of the ricin type.

Alternatively, we provide a recombinant DNA molecule containing a DNA sequence coding for at least a portion of the A chain of a plant toxin of the ricin type said portion coding for a polypeptide that displays the ribosome-inactivity property of the A chain of ricin.

According to a further aspect of the present invention we provide a genetically modified host micro-organism which contains a recombinant DNA molecule as hereinbefore defined.

In the recombinant DNA molecules according to the present invention, the nucleotide sequence coding for the B chain may be modified to eliminate or inactivate the galactose binding sites, and the precursor polypeptide and hence in the mature protein, to eliminate or inactivate the galactose binding sites, and the sequences encoding the signals for N-glycosylation may also be modified to render them ineffective or to eliminate them. Examples of techniques which may prove useful are deletion or oligonucleotide mediated mutagenesis.

The host organism may be a plant cell or an animal cell or preferably a microorganism.

The microorganism may be a prokaryote or a eukaryote. As examples of prokaryotes may be mentioned Gram-negative bacteria, e.g. *E. coli, Methylophilus, methylotrophus* and *Alcaligenes eutrophus*; and Gram-positive bacteria, e.g. *Streptomyces, Bacillus subtilis* and *Arthrobacter*. As examples of eukaryotes may be mentioned yeasts, for example *Saccharomyces cerevisiae*.

The recombinant DNA molecules may comprise a cloning vector such as a plasmid or phage vector into which has been inserted the DNA sequence coding for at least a prortion of a precursor polypeptide, or at least a portion of either the A chain or the B chain, of a ricin-type plant toxin.

The cloning vector is preferably a plasmid although we do not exclude the possibility that it may be a phage vector. The plasmid may be a naturally-occurring plasmid or preferably a composite derived from fragments of other plasmids. Where a composite plasmid is employed it preferably contains promoter sequences which improve expression of the ricin gene.

Examples of suitable plasmids which may be used as cloning vehicles are *inter alia* for Gram-negative bacteria: pBR322, pAT153, pUC8, pGSS15 and pMB9; for Gram-positive bacteria: pVC6; and for *S.cerevisiae:* pMA91, pMA230, YRp7, pLC544 and YEp6. The vector will be selected to be suitable for the particular host envisaged.

We also provide a method of obtaining a recombinant DNA molecule which comprises preparing a double-stranded DNA sequence which codes for a polypeptid sequence present in a plant toxin of th ricin type and inserting said double-stranded DNA sequence into a cloning vector.

More specifically such a method may c mprise isolating the mRNA which codes for the ricin A and B

chain precursor polypeptide, synthesising single-stranded cDNA from said mRNA using reverse transcriptase and a suitable primer, assembling the second DNA strand onto the template formed by said first strand by means of DNA polymerase followed by S1 nuclease, and inserting the resulting double-stranded cDNA into a cloning vector.

Alternatively, the cDNA assembled from the mRNA may be cut into separate portions which code respectively for separate portions of the ricin molecule, for example for the A and B chains, which portions are then inserted into separate cloning vectors.

As stated above the cloning vector is preferably a plasmid such as pBR322, pAT153, of pUC8, and this may be cut open by means of the restriction endonuclease *Pst* I, and may be tailed with oligo (dG), and annealed with the double-stranded cDNA which has been tailed with oligo (dC).

We also provide a method of producing a modified transformed host by introducing into a suitable host microorganism a recombinant DNA molecule according to this invention.

The microorganism used as the host for cloning is preferably a Gram-negative bacterium and more preferably *E. coli*.

After cloning, the DNA sequence coding for the ricin precursor (or the precursor of another ricin-type toxin which is formed from a precusor) may be removed from the host cloning vector. It may then be divided into two portions which code for separate regions of the toxin molecule, for example the A and B chains, these portions introduced into separate second cloning vectors and new hosts modified with each of the resulting new recombinant DNA molecules. Alternatively it may be introduced whole into a second cloning vector. The second cloning vectors comprise suitable promoter sequences and the position and direction of insertion of the whole coding sequence or portion thereof into the second cloning vector are such that on introducing the new recombinant DNA molecules into suitable host microorganisms, e.g. *E. coli* or *S.cerevisiae*, expression of the desired gene sequence is obtained.

The preparation of a transformed host containing a DNA sequence which codes for the ricin A and B chain precursor polypeptide will now be described by way of example, first in general terms and then in detail. This process is summarised in the accompanying diagram.

Firstly, the mRNA encoding this precursor was enriched in known manner by sucrose density gradient centrifugation. The corresponding cDNA was assembled in single-strand form onto this mRNA in known manner using the enzyme reverse transcriptase, a growing point having first been provided on the mRNA using oligo (dT) as primer which binds onto the polyadenylated 3'-terminus of the mRNA. The immediate product of this reaction is a DNA—RNA hybrid. The RNA strand is removed by hydrolysis, leaving the single-strand DNA intact. This is converted to the double stranded form using the enzyme DNA polymerase in the presence of free nucleotides, which results in a hairpin-shaped molecule, the curved end of which is then removed by the single-strand specific nuclease S1. The resulting double strand cDNA is then tailed with oligo (dC) using terminal transferase, size fractionated to remove small molecules, or vice versa and annealed with the pBR322 or pAT153 vector which has been cut open with *Pst* I and tailed with oligo (dG) using terminal transferase, the cytosine tails on the DNA base pairing with the guanine tails on the vector.

The resulting chimaeric plasmids containing the DNA segment coding for the ricin precusor polypeptide were then used to transform *E-coli* DH1 cells, and the presence of the chimaeric plasmid was ensured by selecting cells displaying tetracycline resistance and ampicillin sensitivity. Over 1600 Tet^r, Amp^s clones were obtained. Colonies derived from each clone were transferred to nitrocellulose filters and clones containing the desired DNA sequence identified using a 32P-end labelled 20 mer oligonucleotide probe, having the DNA sequence $ACCTACAA^A_GTT^C_TTT^A_GCT^A_GCC$ which hybridises to DNA containing the complementary sequence $TGGATGTT^T_CAA^G_AAA^T_CGA^T_CGG$. As the ricin precursor polypeptide have been found to contain the amino sequence — Trp-Met-Phe-Lys-Asn-Asp-Gly- DNA sequence responsible for this is known from the genetic code to be the latter mentioned above.

Using appropriate hybridisation and wash conditions, e.g. as described by Singer-Sam *et al* in (1983) Proc. Natl. Acad. Sci. (U.S.A.), Vol. 80 pp 802—806, 80 clones were selected as positively containing the desired DNA sequence, and of these, the eight largest in the plasmid pBR322 have been initially chosen for further characterisation. Their relationship to the castor bean lectin precursor polypeptides has been confirmed using the hybrid release translation assay. Of the eight clones mentioned above, four respectively with 1614, 1950, 1059 and 1020 base pairs, have been selected for sequencing.

In detail, the transformed host was prepared as follows:

A. cDNA synthesis

1. mRNA extraction and fractionation

100—200 g of ripening *Ricinus* seeds were frozen and ground to a powder in liquid nitrogen, and homogenised in a Waring blender for 1—2 minutes in 50 mM tris-HCl pH 9, 150 mM NaCl, 5 mM EDTA and 5% SDS. The homogenate was extracted with an equal volume of phenol:chloroform (1:1) and the phases were separated by centrifugation. The organic phase and residue were reextracted with 0.5 volume of 20 mM tris-HCl pH 9.0, 2 mM EDTA and the resultant aqueous phase was combined with the original one. The total aqueous phase was reextracted repeatedly with equal volumes of phenol:chloroform until no material was present at the interface. RNA was precipitated by the addition of 2 volumes of cold ethanol after making the solution 200 mM in NaCl.

After overnight precipitation at −20°C the RNA was centrifuged at 10,000 rpm for 30 minutes in an MSE 18 or MSE 21 centrifuge; the pellet was then washed repeatedly in 3 M NaAc pH 5.5 until no polysaccharide was detectable in the supernatant by ethanol precipitation. The final pellet was dissoved in 300 mM NaCl and precipitated as above.

mRNA molecules bearing poly(A) tails were extracted by affinity chromatography on olig (dT)-cellulose: after hybridisation at room temperature for 30 min. in 400 mM NaCl, 20 mM tris-HCl pH 7.6, 0.2% SDS, the beads were pelleted and washed three times in the above buffer and two times in 200 mM NaCl, 20 mM tris-HCl pH 7.6, 0.1% SDS. The slurry was poured into a column and washed further with the last buffer until the A_{260} of the eluate reached the background level. Poly(A)-containing RNA was then eluted with 20 mM tris-HCl pH 7.6 at 50°C. The eluate was monitored with an ISCO continuous flow UV cell. Poly(A)-containing RNA was precipitated overnight from 200 mM NaCl by the addition of 2 volumes of cold ethanol at −20°C and was then washed three times with 70% ethanol, and redissolved in 10 mM tris-HCl pH 7.0 to approximately 1 µg/µl.

The mRNA was heated for 2 min. at 65°C and quench cooled. Approximately 400 µg of poly(A)$^+$ RNA was layered on top of a 10—30% ribonuclease-free sucrose (Sigma) density gradient in 100 mM tris-HCl pH 7.5, 0.5% SDS, 1 mM EDTA, and centrifuged in a Beckman L5—65B centrifuge using an SW27 rotor at 25,000 rpm at 17°C for 14 hours. 400 µl fractions were collected with an ISCO density gradient fractionator using the continuous flow UV cell.

Each fraction was made 200 mM in NaCl and precipitated with 2 volumes of cold ethanol by freeze-thawing in liquid nitrogen three times, and recovered by centrifugation in an Eppendorf microcentrifuge for 30 minutes at 4°C, washed once with 70% ethanol, and redissolved in 10 µl of 10 mM tris-HCl pH 7.0. An aliquot from each fraction (1 µl) was translated in a reticulocyte lysate cell free system and the lectin precursor immunoprecipitated to identify the fraction enriched for lectin mRNA.

2. First strand synthesis

Fractionated poly(A)$^+$ RNA was reverse transcribed at 0.5 µg/µl in the presence of 50 mM tris-HCl pH 8.3, 10 mM $MgCl_2$, 100 mM KCl, 1 mM of dATP, dTTP and dGTP, 250 µM dCTP, 0.06 µg/µl oligo$(dT)_{12-18}$, 10 mM DTT and 0.4 units/µl of reverse transcriptase from avian myeloblastosis virus. (3H)dCTP or α-(^{32}P)dCTP were included in the reaction as appropriate.

The reaction mixture was incubated at 42°C for 45 minutes, at which point an equal volume of 5 mM tris-HCl pH 8.3, 5 mM DTT, 250 µM dCTP was added along with the same amount of enzyme as previously. The reaction was incubated for a further 45 minutes at 45°C and terminated by freezing. Aliquots were analysed on 1% denaturing agarose gels along with the products of the second strand and S_1 nuclease reactions.

3. Second strand synthesis

mRNA—cDNA hybrids were denatured by boiling the first strand reaction for 3 minutes and cooling rapidly. After pelleting insoluble material in the Eppendorf microfuge for 2 minutes the supernatant was transferred to a fresh chilled tube. For the standard reaction, reagents were added as follows, ignoring elements already present: dATP, dGTP and dTTP to 100 µM, Hepes-KOH pH 6.9 to 105 mM, KCl to 92 mM, dCTP, labelled as appropriate, to 80 µM, and 0.1 units/µl of DNA polymerase. The reaction was allowed to proceed at 20°C for 6 hours, at which time cDNA was removed from the mixture by gel filtration on 1 ml columns of Bio-Gel P60 in 10 mM tris-HCl pH 7.6, 20 mM NaCl, 1 mM EDTA. Fractions were monitored by Cerenkov or liquid scintillation counting, and peak excluded fractions were pooled and precipitated from 0.3 M NaAc pH 6 by the addition of 2 volumes of cold ethanol. Precipitates were recovered by centrifugation in the Eppendorf microcentrifuge for 30 minutes in the cold, and dissolved in water to about 2.5 µg/µl of RNA — equivalent material.

4. S_1 nuclease digestion

Single-stranded regions of double-stranded cDNA were digested with S_1 nuclease from *Aspergillus oryzae*, in the presence of 300 mM NaCl, 30 mM NaAc pH 4.5, 3 mM $ZnCL_2$. The reaction was incubated for 15 minutes at 37°C and then for 15 minutes at 15°C, and was terminated by the addition of tris-HCl pH 7.6 to 130 mM and EDTA to 10 mM; it was then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated from 300 mM NaAc pH 6 with 2 volumes of cold ethanol. The precipitate was dissolved in 10 mM tris-HCl pH 8, 0.1 mM EDTA to 0.25 µg/µl RNA equivalent.

5. Addition of homopolymer tails to DNA

Double-stranded DNA was tailed using terminal transferase with dCTP at 0.001—0.01 µg/µl in the presence of 140 mM potassium cacodylate pH 7.6, 30 mM tris base, 0.1 mM DTT, 1 mM $CoCl_2$ and (^{3}H) or (^{32}P) — labelled dCTP in 75—150 fold excess over 3' termini. The reaction was carried out at 37°C for 6 minut s. The extent of incorporation of label was followed by assaying the amount f TCA — insoluble radioactivity as a proportion of the total radioactivity, counting in Bray's scintillant.

The reaction was stopped by chilling and adding EDTA to 10 mM, after which unicorporated material was removed by gel filtration as described. Tailed cDNA was precipitated as before, and dissolved in 1 M NaAc pH 8, 10 mM tris-acetate pH 8, 1 mM EDTA ready for fractionation.

PstI-cleaved pBR322 DNA was similarly treated, except that dGTP replaced dCTP.

6. Fracti nati n of tailed cDNA

cDNA was fractionated on 5—20% linear sucrose density gradients in 1 M NaAc pH 8, 10 mM tris-acetate pH 8, 1 mM EDTA, and centrifuged overnight at 39,000 rpm in an SW50.1 rotor. DNA s dimentation was checked on a parallel gradient loaded with a mixture of HinfI and PstI digests of pBR322 DNA, and fractions of this gradient were run on a 1% neutral agarose gel. Fractions from the cDNA gradient were diluted with an equal volume of water and precipitated with 2 volumes of cold ethanol, and then pooled to give three final fractions, a large cDNA fraction (larger than 2,200 bp), an intermediate fraction (1,000—2,200 bp) and a fraction containing smaller cDNAs (600—1,000 bp). cDNA molecules smaller than 600 bp were discarded.

The three final fractions were dissolved to approximately 5 ng/µl in 150 mM RbCl, 10 mM tris-HCl pH 7.6, 0.2 mM EDTA.

B. Annealing and transformation

1. Annealing

dC-tailed cDNA was mixed with dG-tailed pBR322 or pAT153 in approximately equimolar quantities, at a concentration of 0.4 ng/µl of vector. Buffers were as described above. The mixtures were heated to 70°C for 30 minutes and then cooled overnight to room temperature, and slowly chilled to 4°C. Competent cells were added and transformed as described below.

2. Preparation of competent cells and transformation

DHI cells (recAl, nalA, r_k^-, m_k^+, endol$^-$, R$^-$, relAl?) were grown in 10 ml cultures of psi broth (2% tyrptone, 0.5% yeast extract, 10 mM NaCl, 20 mM $MgCl_2$, pH adjusted to 7.6 with KOH; all bacteriological reagents from Difco), and grown at 37°C in a shaking waterbath to A_{550} = 0.3. 1 ml of this was then inoculated into 25 ml of the same medium and grown to A_{550} = 0.48. The cells were then chilled on ice for 15 minutes and harvested at 5,000 rpm for 5 minutes in an MSE 21 centrifuge at 4°C. They were then resuspended in 10 ml of 100 mM RbCl, 50 mM $McCl_2$, 10 mM $CaCl_2$, 35 mM NaAc pH 5.8, 15% glycerol and kept on ice for 15 minutes.

The cells were again harvested, and resuspended in 1 ml of 10 mM RbCl, 75 mM $CaCl_2$, 10 mM MOPS—KOH pH 5.8, 15% glycerol, and kept on ice for a further 15 minutes.

100 µl of the cells thus prepared were mixed with the annealed DNA samples, and incubated on ice for 30 minutes, after which they were heat-shocked at 42°C for 90—120 seconds. 1 ml of psi broth was added, and the cells were grown at 37°C for 1 hour. They were then centrifuged briefly, and resuspended in 100 µl of psi broth and plate on LB plates containing 14 µg/ml tetracycline (LB is 1% tryptone, 0.5% yeast extract, 170 mM NaCl, 1.5% agar).

After 18—24 hours growth at 37°C, colonies were counted and spotted onto LB plates containing 33 µg/ml ampicillin to identify those transformants containing recircularised or uncut plasmids. Over 1600 Tet^r Amp^s clones were picked and transferred in ordered arrays onto large plates of LB containing 14 µg/ml tetracycline.

C. Screening

1. Labelling of oligonucleotide

The ricin B chain specific oligomer (20 mer) was end labelled using polynucleotide kinase. 500 ng of oligonucleotide was incubated in 50 mM tris pH 8.5, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine-HCl, 0.1 mM EDTA with 60 µCi $\gamma(^{32}p)$ ATP and 1 µl polynucleotide kinase (Boehringer) for 35 min at 37°C. The reaction was stopped by adding an equal volume of 0.6 M NH_4AC and the bulk of non incorporated γATP was removed by passage through a sephadex G25 column in 0.14 M NaCl, 0.02 M tris pH 7.6, 0.005 M EDTA, 0.1% SDS. The probe was stored frozen at −20°C.

2. Colony Hybridization using oligonucleotide probe

Transformants were grown on nitrocellulose filters (Schleicher & Schuell 0.45 µ) layered over LB plus tetracycline. The filters, in triplicate, were then transferred to LB-Tet plates containing 200 µg/ml chloramphenicol for 16 h at 37°. The filters were placed colony side up on two sheets of 3 mm paper wetted with 0.5 M NaOH for 15 mins at room temperature. The same procedure was followed for the following two washes (1) with 1 M tris pH 8.0, and (2) with 1 M tris pH 8, 1.5 M NaCl (30 mins). The filters were air dried and baked at 80°C.

Prehybridizations and hybridizations were done in double sealed polythene bags. The filters were prehybridized in 0.9 M NaCl, 0.09 M tris 7.4, 0.006 M EDTA, 0.5% NP40, 2 × Denhardts, 0.2% SDS, 100 µg/ml denatured single strand salmon sperm DNA and 70 µg/ml tRNA. Prehybridization was done for 4 h at 55°C. The prehybridation buffer was then squeezed from the bag and fresh buffer add d that contained 50 ng labelled probe (to give a maximum concentration of 5 ng/ml buffer). Annealing was done overnight at 37°C.

Non stringent washes were done with 6 × SSC at room temperature. The filters were washed in 4 changes of 6 × SSC over 3 h. The triplicate filters were then washed at three different temperatures determined from the base c mositi n and degree of mismatch of the prob . Using 2°C for every A or T

and 4°C for every C or G in the probe the wash temperatures selected were 52°C, 56°C and 60°C. The filters were washed at the stringent temperature in 6 × SSC for 10 minutes and then dried thoroughly. The filters were exposed to X-ray film overnight.

D. Hybrid selection procedure

1. DNA binding

Plasmid DNA was purified from the positive clone(s) and 10—15 µg linearized with EcoRI. After phenol:chloroform extraction and ethanol precipitation the pellet was dissolved in 0.5 ml 0.1 × SSC. 0.5 ml 1 M NaOH was then added and the mixture allowed to stand for 15 mins at room temperature. 4 ml of a prechilled neutralizing solution (1.5 M NaCl, 0.25 M HCl, 0.25 M tris-(λpH 8.0) was added and the 5 ml DNA sample sucked by vacuum through swinnies containing wetted Schleicher and Schuell 0.45 µ filter discs. 5 ml 6 × SSC was then passed through the filter(s). These were air dried and then baked for 2 h at 80°C.

2. Hybrid selection protocol

The filter(s) were placed in 5 ml bottles and prehybridized for 4 h at 41°C in 50% formamide, 0.4 M NaCl, 10 mM pipes-NaOH pH 6.4, 4 mM EDTA, 0.5 µg/ml tRNA, 10 µg/ml poly (A). The buffer was removed and the filter(s) typically hybridized overnight at 41°C in 50% formamide buffer (above) containing approx. 20 µg poly $(A)^+$ RNA from castor beans. The buffer was removed and the filters washed twice for 15 mins in each of the following: (1) 1 × SSC, 0.5% SDS at room temperature, (2) 0.1 × SSC, 0.1% SDS at room temperature, (3) 0.1 × SSC, 0.1% SDS at 50°C, (4) 0.1 × SSC, 0.1% SDS at room temperature. The filters were drained and 200 µl hybrid release buffer (90% formamide, 10 mM pipes-NaOH pH 6.4, 1 mM EDTA, 0.5% SDS) added to each and mixed for 30 mins at 40°C. The buffer was removed into a fresh eppendorf and NaCl added to .2 M. The released mRNA was precipitated with ethanol, rinsed several times in 70% ethanol, dried and dissolved in 5 µl sterile water. The sample was translated in a reticulocyte lysate cell free system and the products run out directly on an SDS-polyacrylamide gel or firstly immunoprecipitated with appropriate antisera.

The DNA sequence coding for the above-mentioned ricin precursor polypeptide of two of the above-mentioned clones, referred to herein as pBRCL 6 and pBRCL 17 (RCL = Ricinus communis lectin) has now been determined by a combination of the Sanger dideoxy method (Sanger et al, 1977 — Proc. Natl. Acad. Sci. U.S.A. *74*, 5463—67) and the procedure of Maxam and Gilbert (Maxam and Gilbert, 1980 — Meth. Enzym. *65*, 499—560). In order to determine the sequence at the ends of each insert, the inserts were excised from pBR322 with Pst I and ligated into Pst I linearized, phosphatased plasmid pUC8 (Vierra and Messing, 1982 — Gene 19, 259—268). E. Coli DHI cells were transformed by these recombinant plasmids. These new recombinant plasmids are referred to herein as pRCL6 and pRCL17.

It is apparent that the two inserts contain a region of common sequence and that together they represent a total ricin precusor sequence. There are no nucleotide differences between the overlapping regions of the inserts in pRCL6 and pRCL17.

A new recombinant DNA molecule was then constructed that contains the complete nucleotide sequence encoding the ricin precursor polypeptide. This was achieved by isolating a fragment, 323 base pairs in length, obtained from pRCL17 by digestion with the restriction endonuclease Sau 961, and ligating this fragment to a fragment 1561 base pairs in length isolated after a partial digestion of pRCL6 with Sau 961. Ligation was performed in 50 mM tris HCl (pH 7.4) 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM spermidine, 10 mM ATP, 0.1 mg/ml BSA with 5 units commercial T4 DNA ligase, and incubation proceeded overnight at 15°C. After a standard phenol/chloroform extraction and ethanol precipitation the ligated DNA was pelleted, dissolved in a small volume of 10 mM tris HCl (pH 7.4), 1 mM EDTA and digested to completion with Pst I. The resulting linearised DNA was then ligated (as above) with an equal quantity of Pst I linearised, phosphatased pUC8. The new recombinant DNA molecule containing the entire DNA sequence of the ricin precusor and referred to as pRCL617, was used in conventional manner to transform *E. coli* DHI cells.

The nucleotide sequence of pRCL617 is shown hereinafter.

This sequence was deduced from the two overlapping cDNA inserts in clones pRCL6 and pRCL17 (the limits of the DNA inserts in each of these two clones are given below).

Nucleotide residues are numbered in the 5' to 3' direction with the first residue of the codon specifying the amino terminal residue of mature ricin A chain numbered 1 and the nucleotides on the 5' side of residue 1 indicated by negative numbers. The 5' terminal sequence does not extend to the 5' end of the mRNA whereas the 3' terminal sequence shown is followed by a poly (dA) tract 27 residues long, thus representing the complete sequence of the region. The predicted amino acid sequence is given below the nucleotide sequence and differences with the published amino acid sequence of mature ricin A and B chains (Funatsu G., Kimura, M. and Funatsu, M. Agric. Biol. Chem. Vol 43, pp 2221—2224 (1979), and Yoshitake, S., Funatsu, G. and Funatsu, M. — Agric. Biol. Chem. Vol. 42, pp 1267—1274 (1978)) are indicated und meath. Residues absent from the published amino acid sequence are underlined with a dashed line and the position of amino acids present in the published sequence but absent from the derived sequence pres nted here are indicated by an asterisk. The dashed line beneath the 12 amino acid sequence linking the C-terminus of the A chain and the N-terminus of the B chain is bracketed. Amino acids are numbered from the amino terminal residue of th mature A chain and the preceding residu s are indicated by

negative numbers. Potential sites for asparagine linked N-glycosylation are boxed and potential poly (A) signals are underlined. The insert of pRCL6 extends from nucleotide — 102 to residue 1512 and the insert of pRCL17 extends from nucleotide 733 to residue 1782.

The intervening twelve triplets code for the linker amino acid sequence which is present in the precursor polypeptide and which is enzymatically removed in the cell to separate the A and B chains, which chains are joined by a disulphide bridge during the formation of the ricin molecule itself. This linker region as well as the presumptive amino terminal leader or signal sequence (amino acids −24 to −1) are not present in the sequences already publised by Funatsu *et al.*

Preproricin is the whole polypeptide coded for the aforesaid DNA insert, i.e. from amino acid −24 to amino acid 541. Proricin, which is obtained from preproricin in the organism by removal of the amino acid leader sequence, extends from amino acid 1 to amino acid 541.

−100 −50
5'-AAACCGGGAG GAAATACTAT TGTAATATGG ATG TAT GCA GTG GCA ACA TGG CTT
Met Tyr Ala Val Ala Thr Trp Leu
−20

−1 1
TGT TTT GGA TCC ACC TCA GGG TGG TCT TTC ACA TTA GAG GAT AAC AAC ATA
Cys Phe Gly Ser Thr Ser Gly Trp Ser Phe Thr Leu Glu Asp Asn Asn Ile
−10 −1 1

50
TTC CCC AAA CAA TAC CCA ATT ATA <u>AAC TTT ACC</u> ACA GCG GGT GCC ACT GTG
Phe Pro Lys Gln Tyr Pro Ile Ile [Asn Phe Thr] Thr Ala Gly Ala Thr Val
10

100
CAA AGC TAC ACA AAC TTT ATC AGA GCT GTT CGC GGT CGT TTA ACA ACT GGA
Gln Ser Tyr Thr Asn Phe Ile Arg Ala Val Arg Gly Arg Leu Thr Thr Gly
20 30

150
GCT GAT GTG AGA CAT GAT ATA CCA GTG TTG CCA AAC AGA GTT GGT TTG CCT
Ala Asp Val Arg His Asp Ile Pro Val Leu Pro Asn Arg Val Gly Leu Pro
40 Glu 50

200
ATA AAC CAA CGG TTT ATT TTA GTT GAA CTC TCA AAT CAT GCA GAG CTT TCT
Ile Asn Gln Arg Phe Ile Leu Val Glu Leu Ser Asn His Ala Glu Leu Ser
60 Gln

250
GTT ACA TTA GCC CTG GAT GTC ACC AAT GCA TAT GTG GTC GGC TAC CGT GCT
Val Thr Leu Ala Leu Asp Val Thr Asn Ala Tyr Val Val Gly Tyr Arg Ala
70 Ser 80

300
GGA AAT AGC GCA TAT TTC TTT CAT CCT GAC AAT CAG GAA GAT GCA GAA GCA
Gly Asn Ser Ala tyr Phe Phe His Pro Asp Asn Gln Glu Asp Ala Glu Ala
90 100

350
ATC ACT CAT CTT TTC ACT GAT GTT CAA AAT CGA TAT ACA TTC GCC TTT GGT
Ile Thr His Leu Phe Thr Asp Val Gln Asn Arg Tyr Thr Phe Ala Phe Gly
110 120

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                                                                  400
GGT AAT TAT GAT AGA CTT GAA CAA CTT GCT GGT AAT CTG AGA GAA AAT ATC
Gly Asn Tyr Asp Arg Leu Glu Gln Leu Ala Gly Asn Leu Arg Glu Asn Ile
                                    130

                                                              450
GAG TTG GGA AAT GGT CCA CTA GAG GAG GCT ATC TCA GCG CTT TAT TAT TAC
Glu Leu Gly Asn Gly Pro Leu Glu Glu Ala Ile Ser Ala Leu Tyr Tyr Tyr
        140                                     150

                                                          500
AGT ACT GGT GGC ACT CAG CTT CCA ACT CTG GCT CGT TCC TTT ATA ATT TGC
Ser Thr Gly Gly Thr Gln Leu Pro Thr Leu Ala Arg Ser Phe Ile Ile Cys
                    160                                 170

                                                      550
ATC CAA ATG ATT TCA GAA GCA GCA AGA TTC CAA TAT ATT GAG GGA GAA ATG
Ile Gln Met Ile Ser Glu Ala Ala Arg Phe Gln Tyr Ile Glu Gly Glu Met
                                180

                                                  600
CGC ACG AGA ATT AGG TAC AAC CGG AGA TCT GCA CCA GAT CCT AGC GTA ATT
Arg Thr Arg Ile Arg Tyr Asn Arg Arg Ser Ala Pro Asp Pro Ser Val Ile
    190                                     200

                                              650
ACA CTT GAG AAT AGT TGG GGG AGA CTT TCC ACT GCA ATT CAA GAG TCT AAC
Thr Leu Glu Asn Ser Trp Gly Arg Leu Ser Thr Ala Ile Gln Glu Ser Asn
                210                                 220

                                          700
CAA GGA GCC TTT GCT AGT CCA ATT CAA CTG CAA AGA CGT AAT GGT TCC AAA
Gln Gly Ala Phe Ala Ser Pro Ile Gln Leu Gln Arg Arg Asn Gly Ser Lys
                            230                 ---  Asp

                                      750
TTC AGT GTG TAC GAT GTG AGT ATA TTA ATC CCT ATC ATA GCT CTC ATG GTG
Phe Ser Val Tyr Asp Val Ser Ile Leu Ile Pro Ile Ile Ala Leu Met Val
240                                     Leu 250             ---

                                  800
TAT AGA TGC GCA CCT CCA CCA TCG TCA CAG TTT TCT TTG CTT ATA AGG CCA
Tyr Arg Cys Ala Pro Pro Pro Ser Ser Gln Phe Ser Leu Leu Ile Arg Pro
            260                                 (---  ---  ---  ---  ---  ---

                                              850
GTG GTA CCA AAT TTT AAT GCT GAT GTT TGT ATG GAT CCT GAG CCC ATA GTG
Val Val Pro Asn Phe Asn Ala Asp Val Cys Met Asp Pro Glu Pro Ile Val
---  ---  ---  ---  ---  ---) 280                                290

                                  900
CGT ATC GTA GGT CGA AAT GGT CTA TGT GTT GAT GTT AGG GAT GGA AGA TTC
Arg Ile Val Gly Arg Asn Gly Leu Cys Val Asp Val Arg Asp Gly Arg Ph
                                300 Asn
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                                                                950
CAC AAC GGA AAC GCA ATA CAG TTG TGG CCA TGC AAG TCT AAT ACA GAT GCA
His Asn Gly Asn Ala Ile Gln Leu Trp Pro Cys Lys Ser Asn Thr Asp Ala
Asn His 310                                         320

                                                            1000
AAT CAG CTC TGG ACT TTG AAA AGA GAC AAT ACT ATT CGA TCT AAT GGA AAG
Asn Gln Leu Trp Thr Leu Lys Arg Asp Asn Thr Ile Arg Ser Asn Gly Lys
          ---     330                                       340

                                                        1050
TGT TTA ACT ACT TAC GGG TAC AGT CCG GGA GTC TAT GTG ATG ATC TAT GAT
Cys Leu Thr Thr Tyr Gly Tyr Ser Pro Gly Val Tyr Val Met Ile Tyr Asp
                        Pro Ser

                                                    1100
TGC AAT ACT GCT GCA ACT GAT GCC ACC CGC TGG CAA ATA TGG GAT
Cys Asn Thr Ala Ala Thr Asp Ala Thr Arg Trp Gln Ile Trp Asp
    360                 Thr      Asp      ---  Glu          Asn

                                                        1150
AAT GGA ACC ATC ATA AAT CCC AGA TCT AGT CTA GTT TTA GCA GCG ACA TCA
Asn Gly Thr Ile Ile Asn Pro Arg Ser Ser Leu Val Leu Ala Ala Thr Ser
                    380                                     390

                                                        1200
GGG AAC AGT GGT ACC ACA CTT ACG GTG CAA ACC AAC ATT TAT GCC GTT AGT
Gly Asn Ser Gly Thr Thr Leu Thr Val Gln Thr Asn Ile Tyr Ala Val Ser
                                    400

                                                    1250
CAA GGT TGG CTT CCT ACT AAT AAT ACA CAA CCT TTT GTT ACA ACC ATT GTT
Gln Gly Trp Leu Pro Thr Asn Asn Thr Gln Pro Phe Val Thr Thr Ile Val
          Pro   Phe                                 Trp 420

                                                1300
GGG CTA TAT GGT CTG TGC TTG CAA GCA AAT AGT GGA CAA GTA TGG ATA GAG
Gly Leu Tyr Gly Leu Cys Leu Gln Ala Asn Ser Gly Gln Val Trp Ile Glu
                    430                             Val 440

                                                1350
GAC TGT AGC AGT GAA AAG GCT GAA CAA CAG TGG GCT CTT TAT GCA GAT GGT
Asp Cys Ser Ser Glu Lys Ala Glu Gln Gln Trp Ala Leu Tyr Ala Asp Gly
    Ser Cys                         450                     Ser

                                                1400
TCA ATA CGT CCT CAG CAA AAC CGA GAT AAT TGC CTT ACA AGT GAT TCT AAT
Ser Ile Arg Pro Gln Gln Asn Arg Asp Asn Cys Leu Thr Ser Asp Ser Asn
Asn 460 Asn                 Arg                 470

                                                1450
ATA CGG GAA ACA GTT GTT AAG ATC CTC TCT TGT GGC CCT GCA TCC TCT GGC
Ile Arg Glu Thr Val Val Lys Ile Leu Ser Cys Gly Pr Ala Ser Ser Gly
                480                             490
```

1500

CAA ĊGA TGG ATG TṪC AAG AAT GAT GGA ACC ATT ṪTA AAT TTG TȦT AGT GGA
Gln Arg Trp Met Phe Lys Asn Asp Gly Thr Ile Leu Asn Leu Tyr S r Gly
Glu 500

1550

TTĠ GTG TTA GAT ĠTG AGG CGA TĊG GAT CCG AGĊ CTT AAA CAA ȦTC ATT CTT
Leu Val Leu Asp Val Arg Arg Ser Asp Pro Ser Leu Lys Gln Ile Ile Leu
510 Ala 520

1600

TȦC CCT CTC CAṪ GGT GAC CCA ȦAC CAA ATA TĠG TTA CCA TTȦ TTT TGA
Tyr Pro Leu His Gly Asp Pro Asn Gln Ile Trp Leu Pro Leu Phe ***
Trp * --- --- *Leu Pro

1650

TAGȦCAGATT ACTĊTCTTGC AGTĠTGTGTG TCCṪGCCATG AAAȦTAGATG GCTṪAAATAA

1700

AAAĠGACATT GTAȦATTTTG TAAĊTGAAAG GACȦGCAAGT TATṪGCAGTC CAGṪATCTAA

1750

TAAĠAGCACA ACTȦTTGTCT TGTĠCATTCT 1780
AAATṪT-Poly(A)

Claims

1. A recombinant DNA molecule characterised in that it includes as an insert DNA coding for the A chain of ricin and having the following nucleotide sequence or a portion thereof coding for a polypeptide that displays the ribosome-inactivity property of the A-chain of ricin:

ȦTA

TTC CCC ȦAA CAA TAC ĊCA ATT ATA AAĊ TTT ACC ACA GCG GGI GCC AĊT GTG

CAA AGĊ TAC ACA AAC ṪTT ATC AGA GĊT GTT CGC GGṪ CGT TIA ACA ȦCT GGA

GCT GȦT GTG AGA CAṪ GAT ATA CCA ĠTG TIG CCA AȦC AGA GTT GGṪ TTG CCT

ATA ȦAC CAA CGG TṪT ATT TTA GTṪ GAA CTC TCA ȦAT CAT GCA GȦG CTT TCT

GTṪ ACA TTA GCC ĊTG GAT GTC AĊC AAT GCA TAṪ GTG GTC GGC ṪAC CGT GCT

GĠA AAT AGC GCȦ TAT TTC TTT ĊAT CCT GAC AȦT CAG GAA GAṪ GCA GAA GCA

ȦTC ACT CAT CṪT TTC ACT GAṪ GTT CAA AAT ĊGA TAT ACA TṪC GCC TTT GGṪ

GGT AAT TAT ĠAT AGA CTT GȦA CAA CTT GCṪ GGT AAT CTG ȦGA GAA AAT AṪC

GAG TTG GGȦ AAT GGT CCA ĊTA GAG GAG GĊT ATC TCA GCĠ CTT TAT TAT ṪAC

AGT ACT GĠT GGC ACT CAĠ CTT CCA ACT ĊTG GCT CGT TĊC TTT ATA ATṪ TGC

ATC CAA ȦTG ATT TCA GȦA GCA GCA AGȦ TTC CAA TAT ȦTT GAG GGA GȦA ATG

CGC ACĠ AGA ATT AGG ṪAC AAC CGG AĠA TCT GCA CCȦ GAT CCT AGC ĠTA ATT

ACA CṪT GAG AAT AGṪ TGG GGG AGA ĊTT TCC ACT GĊA ATT CAA GAĠ TCT AAC

CAA ĠGA GCC TTT GĊT AGT CCA ATṪ CAA CTG CAA ȦGA CGT AAT GĠT TCC AAA

TTĊ AGT GTG TAC ĠAT GTG AGT AṪA TTA ATC CCṪ ATC ATA GCT ĊTC ATG GTG

TȦT AGA TGC GCȦ CCT CCA CCA ṪCG TCA CAG TṪT

or a nucleotide sequence which is equivalent to said sequence or portion by virtue of the degeneracy of the genetic code.

2. A recombinant DNA molecule according to Claim 1 characterised in that it includes as in insert DNA coding for the ricin and having the following nucleotide sequence or a portion thereof coding for a protein that displays the cell binding and ribosome-inactivity property of the ricin:

ȦTA

TTC CCC ȦAA CAA TAC CĊA ATT ATA AAĊ TTT ACC ACA GCG GGI GCC AĊT GTG

CAA AGĊ TAC ACA AAC ṪTT ATC AGA GĊT GTT CGC GGṪ CGT TIA ACA ȦCT GGA

GCT GȦT GTG AGA CAṪ GAT ATA CCA ĠTG TIG CCA AȦC AGA GTT GGṪ TTG CCT

ATA ȦAC CAA CGG TṪT ATT TTA GTṪ GAA CTC TCA ȦAT CAT GCA GȦG CTT TCT

GTṪ ACA TTA GCC ĊTG GAT GTC AĊC AAT GCA TAṪ GTG GTC GGC ṪAC CGT GCT

GĠA AAT AGC GCȦ TAT TTC TTT ĊAT CCT GAC AȦT CAG GAA GAṪ GCA GAA GCA

ȦTC ACT CAT CṪT TTC ACT GAṪ GTT CAA AAT ĊGA TAT ACA TṪC GCC TTT GGṪ

GGT AAT TAT ĠAT AGA CTT GȦA CAA CTT GCṪ GGT AAT CTG ȦGA GAA AAT AṪC

GAG TTG GGȦ AAT GGT CCA ĊTA GAG GAG GĊT ATC TCA GCĠ CTT TAT TAT ṪAC

AGT ACT GĠT GGC ACT CAĠ CTT CCA ACT ĊTG GCT CGT TĊC TTT ATA ATṪ TGC

ATC CAA ȦTG ATT TCA GȦA GCA GCA AGȦ TTC CAA TAT ȦTT GAG GGA GȦA ATG

CGC ACĠ AGA ATT AGG ṪAC AAC CGG AĠA TCT GCA CCȦ GAT CCT AGC ĠTA ATT

ACA CṪT GAG AAT AGṪ TGG GGG AGA ĊTT TCC ACT GĊA ATT CAA GAĠ TCT AAC

CAA GGA GCC TTT GCT AGT CCA ATT CAA CTG CAA AGA CGT AAT GGT TCC AAA

TTC AGT GTG TAC GAT GTG AGT ATA TTA ATC CCT ATC ATA GCT CTC ATG GTG

TAT AGA TGC GCA CCT CCA CCA TCG TCA CAG TTT TCT TTG CTT ATA AGG CCA

GTG GTA CCA AAT TTT AAT GCT GAT GTT TGT ATG GAT CCT GAG CCC ATA GTG

CGT ATC GTA GGT CGA AAT GGT CTA TGT GTT GAT GTT AGG GAT GGA AGA TTC

CAC AAC GGA AAC GCA ATA CAG TTG TGG CCA TGC AAG TCT AAT ACA GAT GCA

AAT CAG CTC TGG ACT TTG AAA AGA GAC AAT ACT ATT CGA TCT AAT GGA AAG

TGT TTA ACT ACT TAC GGG TAC AGT CCG GGA GTC TAT GTG ATG ATC TAT GAT

TGC AAT ACT GCT GCA ACT GAT GCC ACC CGC TGG CAA ATA TGG GAT

AAT GGA ACC ATC ATA AAT CCC AGA TCT AGT CTA GTT TTA GCA GCG ACA TCA

GGG AAC AGT GGT ACC ACA CTT ACG GTG CAA ACC AAC ATT TAT GCC GTT AGT

CAA GGT TGG CIT CCT ACT AAT AAT ACA CAA CCT TTT GTT ACA ACC ATT GTT

GGG CTA TAT GGT CTG TGC TTG CAA GCA AAT AGT GGA CAA GTA TGG ATA GAG

GAC TGT AGC AGT GAA AAG GCT GAA CAA CAG TGG GCT CTT TAT GCA GAT GGT

TCA ATA CGT CCT CAG CAA AAC CGA GAT AAT TGC CTT ACA AGT GAT TCT AAT

ATA CGG GAA ACA GTT GTT AAG ATC CTC TCT TGT GGC CCT GCA TCC TCT GGC

CAA CGA TGG ATG TTC AAG AAT GAT GGA ACC ATT TTA AAT TTG TAT AGT GGA

TTG GTG TTA GAT GTG AGG CGA TCG GAT CCG AGC CTT AAA CAA ATC ATT CTT

TAC CCT CTC CAT GGT GAC CCA AAC CAA ATA TGG TTA CCA TTA TTT

or a nucleotide sequence which is equivalent to said sequence or portion by virtue of the degeneracy of the genetic code.

3. A recombinant DNA molecule according to Claim 1 or Claim 2 characterised in that it includes at the NH_2- terminal end DNA coding for a signal polypeptide having the nucleotide sequence

ATG TAT GCA GTG GCA ACA TGG CTT TGT TTT GCA

TCC ACC TCA GGG TGG TCT TTC ACA TTA GAG

GAT AAC AAC

or a nucleotide sequence which is equivalent thereto by virtue of the degeneracy of the genetic code.

4. A recombinant DNA molecule according to Claim 1, characterised in that it includes as an insert the following DNA sequence which codes for preproricin

ATG TAT GCA GTG GCA ACA TGG CTT

TGT TTI GGA TCC ACC TCA GGG TGG TCT TTC ACA TTA GAG GAT AAC AAC ATA

TTC CCC AAA CAA TAC CCA ATT ATA AAC TTT ACC ACA GCG GGI GCC ACT GTG

CAA AGC TAC ACA AAC TTT ATC AGA GCT GTT CGC GGT CGT TIA ACA ACT GGA

GCT GAT GTG AGA CAT GAT ATA CCA GTG TIG CCA AAC AGA GTT GGT TTG CCT

ATA AAC CAA CGG TTT ATT TTA GTT GAA CTC TCA AAT CAT GCA GAG CTT TCT

GTT ACA TTA GCC CTG GAT GTC ACC AAT GCA TAT GTG GTC GGC TAC CGT GCT

GGA AAT AGC GCA TAT TTC TTT CAT CCT GAC AAT CAG GAA GAT GCA GAA GCA

ATC ACT CAT CTT TTC ACT GAT GTT CAA AAT CGA TAT ACA TTC GCC TTT GGT

GGT AAT TAT GAT AGA CTT GAA CAA CTT GCT GGT AAT CTG AGA GAA AAT ATC

GAG TTG GGA AAT GGT CCA CTA GAG GAG GCT ATC TCA GCG CTT TAT TAT TAC

AGT ACT GGT GGC ACT CAG CTT CCA ACT CTG GCT CGT TCC TTT ATA ATT TGC

ATC CAA ATG ATT TCA GAA GCA GCA AGA TTC CAA TAT ATT GAG GGA GAA ATG

CGC ACG AGA ATT AGG TAC AAC CGG AGA TCT GCA CCA GAT CCT AGC GTA ATT

ACA CTT GAG AAT AGT TGG GGG AGA CTT TCC ACT GCA ATT CAA GAG TCT AAC

CAA GGA GCC TTT GCT AGT CCA ATT CAA CTG CAA AGA CGT AAT GGT TCC AAA

TTC AGT GTG TAC GAT GTG AGT ATA TTA ATC CCT ATC ATA GCT CTC ATG GTG

TAT AGA TGC GCA CCT CCA CCA TCG TCA CAG TTT TCT TTG CTT ATA AGG CCA

GTG GTA CCA AAT TTT AAT GCT GAT GTT TGT ATG GAT CCT GAG CCC ATA GTG

CGT ATC GTA GGT CGA AAT GGT CTA TGT GTT GAT GTT AGG GAT GGA AGA TTC

CAC AAC GGA AAC GCA ATA CAG TTG TGG CCA TGC AAG TCT AAT ACA GAT GCA

AAT CAG CTC TGG ACT TTG AAA AGA GAC AAT ACT ATT CGA TCT AAT GGA AAG

TGT TTA ACT ACT TAC GGG TAC AGT CCG GGA GTC TAT GTG ATG ATC TAT GAT

TGC AAT ACT GCT GCA ACT GAT GCC ACC CGC TGG CAA ATA TGG GAT

AAT GGA ACC ATC ATA AAT CCC AGA TCT AGT CTA GTT TTA GCA GCG ACA TCA

GGG AAC AGT GGT ACC ACA CTT ACG GTG CAA ACC AAC ATT TAT GCC GTT AGT

CAA GGT TGG CIT CCT ACT AAT AAT ACA CAA CCT TTT GTT ACA ACC ATT GTT

GGG CTA TAT GGT CTG TGC TTG CAA GCA AAT AGT GGA CAA GTA TGG ATA GAG

GAC TGT AGC AGT GAA AAG GCT GAA CAA CAG TGG GCT CTT TAT GCA GAT GGT

TCA ATA CGT CCT CAG CAA AAC CGA GAT AAT TGC CTT ACA AGT GAT TCT AAT

ATA CGG GAA ACA GTT GTT AAG ATC CTC TCT TGT GGC CCT GCA TCC TCT GGC

CAA CGA TGG ATG TTC AAG AAT GAT GGA ACC ATT TTA AAT TTG TAT AGT GGA

TTG GTG TTA GAT GTG AGG CGA TCG GAT CCG AGC CTT AAA CAA ATC ATT CTT

TAC CCT CTC CAT GGT GAC CCA AAC CAA ATA TGG TTA CCA TTA TTT

or a nucleotide sequence which is equivalent to said sequence or portion by virtue of the degeneracy of the genetic code.

5. A recombinant DNA molecule according to any preceding claim characterised in that said insert has been introduced into a cloning vector which is a plasmid or a bacteriophage to obtain said recombinant DNA molecule.

6. A modified *E. Coli* or *Saccharomyces cervisiae* host cell containing a recombinant DNA molecule according to any preceding claim.

7. A modified host microorganism according to Claim 6, when appendant to Claim 4, wherein the host is *E. Coli,* characterised in that the cloning vector is plasmid selected from pBR322, pAT153, pUC8, pGS15 or pMB9.

8. A modified host microorganism according to Claim 6, when appendant to Claim 4, wherein the host cell is *Sacchoromyces Cervisiae,* characterised in that the cloning vector is pMa91, pMA230, YRp7, pLC544 and YEp6.

9. A meth d of preparing cDNA having a nucleotide sequence as defined in any of Claims 1 t 4 characterised by separating from a mixture of mRNA's obtained from *Ricinus Communis* tissue a m lecular weight fraction containing mRNA coding for preproricin and synthesising cDNA from this by reverse transcription.

10. A method of obtaining a recombinant DNA molecule by inserting a double-stranded cDNA according to Claim 8 into a cloning vector.

11. A method of obtaining a genetically-modified *E. Coli* or *Sacchoromyces cervisiae* host characterised in that a recombinant DNA molecule according to Claim 9 is introduced into said host.

Patentansprüche

1. Ein rekombinantes DNS-Molekül, dadurch gekennzeichnet, daß es als Einfügung DNS enthält, welche die A-Kette des Ricins kodiert und die folgende Nukleotid-Sequenz oder einen Teil davon besitzt, welche ein Polypeptid kodiert, das die Ribosomen-Inaktivitäts-Eigenschaft der A-Kette des Ricins zeigt:

ATA TTC CCC ÀAA CAA TAC CĊA ATT ATA AAĊ TTT ACC ACA GCG GGI GCC AĊT GTG

CAA AGĊ TAC ACA AAC ṪTT ATC AGA GĊT GTT CGC GGṪ CGT TIA ACA ÀCT GGA

GCT GÀT GTG AGA CAṪ GAT ATA CCA ĠTG TIG CCA AÀC AGA GTT GGṪ TTG CCT

ATA ÀAC CAA CGG TṪT ATT TTA GTṪ GAA CTC TCA ÀAT CAT GCA GÀG CTT TCT

GTṪ ACA TTA GCC ĊTG GAT GTC AĊC AAT GCA TAṪ GTG GTC GGC ṪAC CGT GCT

GĠA AAT AGC GCÀ TAT TTC TTT ĊAT CCT GAC AÀT CAG GAA GAṪ GCA GAA GCA

ÀTC ACT CAT CṪT TTC ACT GAṪ GTT CAA AAT ĊGA TAT ACA TṪC GCC TTT GGṪ

GAG TTG GGÀ AAT GGT CCA ĊTA GAG GAG GĊT ATC TCA GCĠ CTT TAT TAT ṪAC

AGT ACT GĠT GGC ACT CAĠ CTT CCA ACT ĊTG GCT CGT TĊC TTT ATA ATṪ TGC

ATC CAA ÀTG ATT TCA GÀA GCA GCA AGÀ TTC CAA TAT ÀTT GAG GGA GÀA ATG

CGC ACĠ AGA ATT AGG ṪAC AAC CGG AĠA TCT GCA CCÀ GAT CCT AGC ĠTA ATT

ACA CṪT GAG AAT AGṪ TGG GGG AGA ĊTT TCC ACT GĊA ATT CAA GAĠ TCT AAC

CAA ĠGA GCC TTT GĊT AGT CCA ATṪ CAA CTG CAA ÀGA CGT AAT GĠT TCC AAA

TTĊ AGT GTG TAC ĠAT GTG AGT AṪA TTA ATC CĊṪ ATC ATA GCT ĊTC ATG GTG

TÀT AGA TGC GCÀ CCT CCA CCA ṪCG TCA CAG TṪT

oder eine Nukleotid-Sequenz, die wegen der Degenerierung des genetischen Kodes dieser Sequenz oder diesem Teil äquivalent ist.

2. Rekombinantes DNS-Molekül nach Anspruch 1, dadurch gekennzeichnet, daß es als Einfügung DNS enthält, die Ricins kodiert und die folgende Nukleotid-Sequenz oder einen Teil davon besitzt, welche ein Protein kodiert, das die Zell-Bindungs- und Ribosomen-Inaktivitäts-Eigenschaft des Ricins zeigt:

ATA TTC CCC ÀAA CAA TAC CĊA ATT ATA AAĊ TTT ACC ACA GCG GGI GCC AĊT GTG

CAA AGĊ TAC ACA AAC ṪTT ATC AGA GĊT GTT CGC GGṪ CGT TIA ACA ÀCT GGA

GCT GAT GTG AGA CAT GAT ATA CCA GTG TIG CCA AAC AGA GTT GGT TTG CCT

ATA AAC CAA CGG TTT ATT TTA GTT GAA CTC TCA AAT CAT GCA GAG CTT TCT

GTT ACA TTA GCC CTG GAT GTC ACC AAT GCA TAT GTG GTC GGC TAC CGT GCT

GGA AAT AGC GCA TAT TTC TTT CAT CCT GAC AAT CAG GAA GAT GCA GAA GCA

ATC ACT CAT CTT TTC ACT GAT GTT CAA AAT CGA TAT ACA TTC GCC TTT GGT

GGT AAT TAT GAT AGA CTT GAA CAA CTT GCT GGT AAT CTG AGA GAA AAT ATC

GAG TTG GGA AAT GGT CCA CTA GAG GAG GCT ATC TCA GCG CTT TAT TAT TAC

AGT ACT GGT GGC ACT CAG CTT CCA ACT CTG GCT CGT TCC TTT ATA ATT TGC

ATC CAA ATG ATT TCA GAA GCA GCA AGA TTC CAA TAT ATT GAG GGA GAA ATG

CGC ACG AGA ATT AGG TAC AAC CGG AGA TCT GCA CCA GAT CCT AGC GTA ATT

ACA CTT GAG AAT AGT TGG GGG AGA CTT TCC ACT GCA ATT CAA GAG TCT AAC

CAA GGA GCC TTT GCT AGT CCA ATT CAA CTG CAA AGA CGT AAT GGT TCC AAA

TTC AGT GTG TAC GAT GTG AGT ATA TTA ATC CCT ATC ATA GCT CTC ATG GTG

TAT AGA TGC GCA CCT CCA CCA TCG TCA CAG TTT TCT TTG CTT ATA AGG CCA

GTG GTA CCA AAT TTT AAT GCT GAT GTT TGT ATG GAT CCT GAG CCC ATA GTG

CGT ATC GTA GGT CGA AAT GGT CTA TGT GTT GAT GTT AGG GAT GGA AGA TTC

CAC AAC GGA AAC GCA ATA CAG TTG TGG CCA TGC AAG TCT AAT ACA GAT GCA

AAT CAG CTC TGG ACT TTG AAA AGA GAC AAT ACT ATT CGA TCT AAT GGA AAG

TGT TTA ACT ACT TAC GGG TAC AGT CCG GGA GTC TAT GTG ATG ATC TAT GAT

TGC AAT ACT GCT GCA ACT GAT GCC ACC CGC TGG CAA ATA TGG GAT

AAT GGA ACC ATC ATA AAT CCC AGA TCT AGT CTA GTT TTA GCA GCG ACA TCA

GGG AAC AGT GGT ACC ACA CTT ACG GTG CAA ACC AAC ATT TAT GCC GTT AGT

CAA GGT TGG CIT CCT ACT AAT AAT ACA CAA CCT TTT GTT ACA ACC ATT GTT

GGG CTA TAT GGT CTG TGC TTG CAA GCA AAT AGT GGA CAA GTA TGG ATA GAG

GAC TGT AGC AGT GAA AAG GCT GAA CAA CAG TGG GCT CTT TAT GCA GAT GGT

TCA ATA CGT CCT CAG CAA AAC CGA GAT AAT TGC CTT ACA AGT GAT TCT AAT

ATA CGG GAA ACA GTT GTT AAG ATC CTC TCT TGT GGC CCT GCA TCC TCT GGC

CAA CGA TGG ATG TTC AAG AAT GAT GGA ACC ATT TTA AAT TTG TAT AGT GGA

TTG GTG TTA GAT GTG AGG CGA TCG GAT CCG AGC CTT AAA CAA ATC ATT CTT

TAC CCT CTC CAT GGT GAC CCA AAC CAA ATA TGG TTA CCA TTA TTT

oder eine Nukleotid-Sequenz, die wegen der Degenerierung des genetischen Kodes dieser Sequenz oder diesem Teil äquivalent ist.

3. Rekombinantes DNS-Molekül nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß es am NH_2-terminalen Ende DNS enthält, welche eine Signal-Polypeptid kodiert, welches die Nukleotid-Sequenz

ATG TAT GCA GTG GCA ACA TGG CTT TGT TTT GCA

TCC ACC TCA GGG TGG TCT TTC ACA TTA GAG

GAT AAC AAC

besitzt oder eine Nukleotid-Sequenz, die wegen der Degenerierung des genetischen Kodes dazu äquivalent ist.

4. Rekombinantes DNS-Molekül nach Anspruch 1, dadurch gekennzeichnet, daß es als Einfügung die folgende DNS-Sequenz enthält, welche Preproricin kodiert:

ATG TAT GCA GTG GCA ACA TGG CTT

TGT TIT GGA TCC ACC TCA GGG TGG TCT TTC ACA TTA GAG GAT AAC AAC ATA

TTC CCC AAA CAA TAC CCA ATT ATA AAC TTT ACC ACA GCG GGI GCC ACT GTG

CAA AGC TAC ACA AAC TTT ATC AGA GCT GTT CGC GGT CGT TIA ACA ACT GGA

GCT GAT GTG AGA CAT GAT ATA CCA GTG TIG CCA AAC AGA GTT GGT TTG CCT

ATA AAC CAA CGG TTT ATT TTA GTT GAA CTC TCA AAT CAT GCA GAG CTT TCT

GTT ACA TTA GCC CTG GAT GTC ACC AAT GCA TAT GTG GTC GGC TAC CGT GCT

GGA AAT AGC GCA TAT TTC TTT CAT CCT GAC AAT CAG GAA GAT GCA GAA GCA

ATC ACT CAT CTT TTC ACT GAT GTT CAA AAT CGA TAT ACA TTC GCC TTT GGT

GGT AAT TAT GAT AGA CTT GAA CAA CTT GCT GGT AAT CTG AGA GAA AAT ATC

GAG TTG GGA AAT GGT CCA CTA GAG GAG GCT ATC TCA GCG CTT TAT TAT TAC

AGT ACT GGT GGC ACT CAG CTT CCA ACT CTG GCT CGT TCC TTT ATA ATT TGC

ATC CAA ATG ATT TCA GAA GCA GCA AGA TTC CAA TAT ATT GAG GGA GAA ATG

CGC ACG AGA ATT AGG TAC AAC CGG AGA TCT GCA CCA GAT CCT AGC GTA ATT

ACA CTT GAG AAT AGT TGG GGG AGA CTT TCC ACT GCA ATT CAA GAG TCT AAC

CAA GGA GCC TTT GCT AGT CCA ATT CAA CTG CAA AGA CGT AAT GGT TCC AAA

TTC AGT GTG TAC GAT GTG AGT ATA TTA ATC CCT ATC ATA GCT CTC ATG GTG

TAT AGA TGC GCA CCT CCA CCA TCG TCA CAG TTT TCT TTG CTT ATA AGG CCA

GTG GTA CCA AAT TTT AAT GCT GAT GTT TGT ATG GAT CCT GAG CCC ATA GTG

CGT ATC GTA GGT CGA AAT GGT CTA TGT GTT GAT GTT AGG GAT GGA AGA TTC

CAC AAC GGA AAC GCA ATA CAG TTG TGG CCA TGC AAG TCT AAT ACA GAT GCA

AAT CAG CTC TGG ACT TTG AAA AGA GAC AAT ACT ATT CGA TCT AAT GGA AAG

TGT TTA ACT ACT TAC GGG TAC AGT CCG GGA GTC TAT GTG ATG ATC TAT GAT

TGC AAT ACT GCT GCA ACT GAT GCC ACC CGC TGG CAA ATA TGG GAT

AAT GGA ACC ATC ATA AAT CCC AGA TCT AGT CTA GTT TTA GCA GCG ACA TCA

GGG AAC AGT GGT ACC ACA CTT ACG GTG CAA ACC AAC ATT TAT GCC GTT AGT

CAA GGT TGG CTT CCT ACT AAT AAT ACA CAA CCT TTT GTT ACA ACC ATT GTT

GGG CTA TAT GGT CTG TGC TTG CAA GCA AAT AGT GGA CAA GTA TGG ATA GAG

GAC TGT AGC AGT GAA AAG GCT GAA CAA CAG TGG GCT CTT TAT GCA GAT GGT

TCA ATA CGT CCT CAG CAA AAC CGA GAT AAT TGC CTT ACA AGT GAT TCT AAT

ATA ĊGG GAA ACA GTT GTT AAG ATC ĊTC TCT TGT GĠC CCT GCA TCĊ TCT GGC

CAA ĊGA TGG ATG TTC AAG AAT GAT GGA ACC ATT ṪTA AAT TTG TAT AGT GGA

TTĠ GTG TTA GAT ĠTG AGG CGA TĊG GAT CCG AGĊ CTT AAA CAA ATC ATT CTT

TAC CCT CTC CAṪ GGT GAC CCA AAC CAA ATA TĠG TTA CCA TTA TTT

oder eine Nukleotid-Sequenz, die wegen der Degenerierung des genetischen Kodes dieser Sequenz oder diesem Teil äquivalent ist.

5. Rekombinantes DNS-Molekül nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß die Einfügung in einen Klonierungs-Zwischenträger, der ein Plasmid oder ein Bakteriophage ist, eingeführt wurde, um das rekombinante DNS-Molekül zu erhalten.

6. Modifizierte E. Coli oder Saccharomyces Cervisiae-Wirtszelle, welche ein rekombinantes DNS-Molekül nach einem der vorhergehenden Ansprüche enthält.

7. Ein modifizierter Wirts-Mikroorganismus nach Anspruch 6 und 4, wobei die Wirts-Zelle E. Coli ist, dadurch gekennzeichnet, daß der Klonierungs-Zwischenträger ein Plasmid, ausgewählt aus pBR322, pAT153, pUC8, pGS15 oder pMB9 ist.

8. Ein modifizierter Wirts-Mikroorganismus nach Anspruch 6 und 4, wobei die Wirts-Zelle Saccharomyces Cervisiae ist, dadurch gekennzeichnet, daß der Klonierungs-Zwischenträger pMA91, pMA230, YRp7, pLC544 and YEp6 ist.

9. Methode zur Darstellung von cDNS mit einer Nukleotid-Sequenz wie in einem der Ansprüche 1 bis 4 definiert, gekennzeichnet durch Abtrennen einer Molekular-gewichtsfraktion, die Preproricin kodierende mRNS enthält, aus einer Mischung von aus Ricinus Communis-Gewebe erhaltenen mRNS und durch Synthetisieren von cDNS aus dieser mittels reverser Transkription.

10. Method, ein rekombinantes DNS-Molekül durch Einsetzen einer doppelsträngigen cDNS nach Anspruch 8 in einen Klonierungs-Zwischenträger zu erhalten.

11. Methode zur Erhaltung eines genetisch modifizierten E. Coli- oder Saccharomyces Cervisiae-Wirts, dadurch gekennzeichent, daß ein rekombinantes DNS-Molekül nach Anspruch 9 in diesen Wirt eingeführt wird.

Revendications

1. Molécule d'ADN recombinant caractérisée en ce qu'elle comprend en tant que segment d'insertion un ADN codant pour la chaîne A de la ricine et ayant la séquence nucléotidique ci-dessus ou une partie de celle-ci codant pour un polypeptide qui manifeste la propriété d'inactivation ribosomale de la chaîne A de la ricine:

ATA

TTC CCC AAA CAA TAC CĊA ATT ATA AAĊ TTT ACC ACA GCG GGI GCC AĊT GTG

CAA AGĊ TAC ACA AAC ṪTT ATC AGA GĊT GTT CGC GGṪ CGT TIA ACA ACT GGA

GCT GAṪ GTG AGA CAṪ GAT ATA CCA ĠTG TIG CCA AAC AGA GTT GGṪ TTG CCT

ATA AAC CAA CGG TTT ATT TTA GTṪ GAA CTC TCA AAT CAT GCA GAG CTT TCT

GTṪ ACA TTA GCC ĊTG GAT GTC AĊC AAT GCA TAṪ GTG GTC GGC ṪAC CGT GCT

GĠA AAT AGC GCA TAT TTC TTT ĊAT CCT GAC AAT CAG GAA GAṪ GCA GAA GCA

ATC ACT CAT CṪT TTC ACT GAṪ GTT CAA AAT ĊGA TAT ACA TTC GCC TTT GGṪ

GGT AAT TAT ĠAT AGA CTT GÀA CAA CTT GCṪ GGT AAT CTG ÀGA GAA AAT AṪC

GAG TTG GGÀ AAT GGT CCA ĊTA GAG GAG GĊT ATC TCA GCĠ CTT TAT TAT ṪAC

AGT ACT GĠT GGC ACT CAĠ CTT CCA ACT ĊTG GCT CGT TĊC TTT ATA ATṪ TGC

ATC CAA ÀTG ATT TCA GÀA GCA GCA AGÀ TTC CAA TAT ÀTT GAG GGA GÀA ATG

CGC ACĠ AGA ATT AGG ṪAC AAC CGG AĠA TCT GCA CCÀ GAT CCT AGC ĠTA ATT

ACA CṪT GAG AAT AGṪ TGG GGG AGA ĊTT TCC ACT GĊA ATT CAA GAĠ TCT AAC

CAÀ ĠGA GCC TTT GĊT AGT CCA ATṪ CAA CTG CAA ÀGA CGT AAT GĠT TCC AAA

TTĊ AGT GTG TAC ĠAT GTG AGT AṪA TTA ATC CCṪ ATC ATA GCT ĊTC ATG GTG

TÀT AGA TGC GCÀ CCT CCA CCA ṪCG TCA CAG TṪT

ou une séquence nucélotidique qui est équivalente à ladite séquence ou partie de la séquence en raison de la dégénescence du code génétique.

2. Molécule d'ADN recombinant selon la revendication 1, caractérisée en ce qu'elle comprend en tant que segment d'insertion un ADN codant pour la ricine et ayant la séquence nucléotidique ci-dessous ou une portion de celle-ci codant pour un protéine qui manifeste la propriété de liaison aux cellules et d'inactivation ribosomale de la ricine:

ÀTA

TTC CCC ÀAA CAA TAC CĊA ATT ATA AAĊ TTT ACC ACA GCG GGI GCC AĊT GTG

CAA AGĊ TAC ACA AAC ṪTT ATC AGA GĊT GTT CGC GGṪ CGT TIA ACA ÀCT GGA

GCT GÀT GTG AGA CAṪ GAT ATA CCA ĠTG TIG CCA AÀC AGA GTT GGṪ TTG CCT

ATA ÀAC CAA CGG TṪT ATT TTA GTṪ GAA CTC TCA ÀAT CAT GCA GÀG CTT TCT

GTṪ ACA TTA GCC ĊTG GAT GTC AĊC AAT GCA TAṪ GTG GTC GGC ṪAC CGT GCT

GĠA AAT AGC GCÀ TAT TTC TTT ĊAT CCT GAC AÀT CAG GAA GAṪ GCA GAA GCA

ÀTC ACT CAT CṪT TTC ACT GAṪ GTT CAA AAT ĊGA TAT ACA TṪC GCC TTT GGṪ

GGT AAT TAT ĠAT AGA CTT GÀA CAA CTT GCṪ GGT AAT CTG ÀGA GAA AAT AṪC

GAG TTG GGÀ AAT GGT CCA ĊTA GAG GAG GĊT ATC TCA GCĠ CTT TAT TAT ṪAC

AGT ACT GĠT GGC ACT CAĠ CTT CCA ACT ĊTG GCT CGT TĊC TTT ATA ATṪ TGC

ATC CAA ÀTG ATT TCA GÀA GCA GCA AGÀ TTC CAA TAT ÀTT GAG GGA GÀA ATG

CGC ACG AGA ATT AGG ṪAC AAC CGG AGA TCT GCA CCÀ GAT CCT AGC ĠTA ATT

ACA CṪT GAG AAT AGṪ TGG GGG AGA ĊTT TCC ACT GĊA ATT CAA GAĠ TCT AAC

CAA ĠGA GCC TTT GĊT AGT CCA ATṪ CAA CTG CAA ÀGA CGT AAT GĠT TCC AAA

TTĊ AGT GTG TAC ĠAT GTG AGT AṪA TTA ATC CCṪ ATC ATA GCT ĊTC ATG GTG

TÀT AGA TGC GCÀ CCT CCA CCA ṪCG TCA CAG TṪT TCT TTG CTṪ ATA AGG CCA

ĠTG GTA CCA AÀT TTT AAT GCṪ GAT GTT TGT ÀTG GAT CCT GÀG CCC ATA GTĠ

CGT ATC GTA GGT CGA AAT GĠT CTA TGT GTṪ GAT GTT AGG ĠAT GGA AGA TṪC

CAC AAC GGÀ AAC GCA ATA ĊAG TTG TGG CĊA TGC AAG TCṪ AAT ACA GAT ĠCA

AAT CAG CṪC TGG ACT TTĠ AAA AGA GAC ÀAT ACT ATT CĠA TCT AAT GGÀ AAG

TGT TTA ÀCT ACT TAC GĠG TAC AGT CCĠ GGA GTC TAT GTG ATG ATC TÀT GAT

TGC AAṪ ACT GCT GCA ÀCT GAT GCC AĊC CGC TGG CAÀ ATA TGG GAT

ÀAT GGA ACC AṪC ATA AAT CCĊ AGA TCT AGT ĊTA GTT TTA GĊA GCG ACA TCÀ

GGG AAC AGT ĠGT ACC ACA CṪT ACG GTG CAÀ ACC AAC ATT ṪAT GCC GTT AĠT

CAA GGT TGĠ CIT CCT ACT ÀAT AAT ACA CÀA CCT TTT GTṪ ACA ACC ATT GTT

GGG CTA TÀT GGT CTG TGĊ TTG CAA GCA ÀAT AGT GGA CÀA GTA TGG ATÀ GAG

GAC TGT ÀGC AGT GAA AÀG GCT GAA CAÀ CAG TGG GCT ĊTT TAT GCA GÀT GGT

TCA ATÀ CGT CCT CAG ĊAA AAC CGA GAT AAT TGC CTṪ ACA AGT GAT ṪCT AAT

ATA ĊGG GAA ACA GṪT GTT AAG ATC ĊTC TCT TGT GĠC CCT GCA TCĊ TCT GGC

CAA ĊGA TGG ATG TṪC AAG AAT GAT GGA ACC ATT ṪTA AAT TTG TÀT AGT GGA

TTĠ GTG TTA GAT ĠTG AGG CGA TĊG GAT CCG AGĊ CTT AAA CAA ÀTC ATT CTT

TÀC CCT CTC CAṪ GGT GAC CCA AAC CAA ATA TĠG TTA CCA TTÀ TTT

ou une séquence nucléotidique qui st équivalente à ladite séquence ou partie de la séquence ou partie de celle-ci en raison de la dégénescence du code génétique.

3. Molécule d'ADN recombinant selon la revendication 1 ou la revendication 2, caractérisée en ce qu'ell compr nd à l'extrémité NH_2-terminale un ADN codant pour un polypeptide de signal ayant la séquence nucléoiditique:

ATG TAT GCA GTG GCA ACA TGG CTT TGT TTT GCA

TCC ACC TCA GGG TGG TCT TTC ACA TTA GAG

GAT AAC AAC

ou une séquence nucléotidique qui est équivalente à celle-ci en raison de la dégénescence du code génétique.

4. Molécule d'ADN recombinant selon la revendication 1, caractérisée en ce qu'elle comprend en tant segment d'insertion la séquence d'ADN suivante qui code pour la préproricine:

ATG TAT GCA GTG GCA ACA TGG CTT

TGT TIT GGA TCC ACC TCA GGG TGG TCT TTC ACA TTA GAG GAT AAC AAC ATA

TTC CCC AAA CAA TAC CCA ATT ATA AAC TTT ACC ACA GCG GGI GCC ACT GTG

CAA AGC TAC ACA AAC TTT ATC AGA GCT GTT CGC GGT CGT TIA ACA ACT GGA

GCT GAT GTG AGA CAT GAT ATA CCA GTG TIG CCA AAC AGA GTT GGT TTG CCT

ATA AAC CAA CGG TTT ATT TTA GTT GAA CTC TCA AAT CAT GCA GAG CTT TCT

GTT ACA TTA GCC CTG GAT GTC ACC AAT GCA TAT GTG GTC GGC TAC CGT GCT

GGA AAT AGC GCA TAT TTC TTT CAT CCT GAC AAT CAG GAA GAT GCA GAA GCA

ATC ACT CAT CTT TTC ACT GAT GTT CAA AAT CGA TAT ACA TTC GCC TTT GGT

GGT AAT TAT GAT AGA CTT GAA CAA CTT GCT GGT AAT CTG AGA GAA AAT ATC

GAG TTG GGA AAT GGT CCA CTA GAG GAG GCT ATC TCA GCG CTT TAT TAT TAC

AGT ACT GGT GGC ACT CAG CTT CCA ACT CTG GCT CGT TCC TTT ATA ATT TGC

ATC CAA ATG ATT TCA GAA GCA GCA AGA TTC CAA TAT ATT GAG GGA GAA ATG

CGC ACG AGA ATT AGG TAC AAC CGG AGA TCT GCA CCA GAT CCT AGC GTA ATT

ACA CTT GAG AAT AGT TGG GGG AGA CTT TCC ACT GCA ATT CAA GAG TCT AAC

CAA GGA GCC TTT GCT AGT CCA ATT CAA CTG CAA AGA CGT AAT GGT TCC AAA

TTC AGT GTG TAC GAT GTG AGT ATA TTA ATC CCT ATC ATA GCT CTC ATG GTG

TAT AGA TGC GCA CCT CCA CCA TCG TCA CAG TTT TCT TTG CTT ATA AGG CCA

GTG GTA CCA AAT TTT AAT GCT GAT GTT TGT ATG GAT CCT GAG CCC ATA GTG

CGT ATC GTA GGT CGA AAT GGT CTA TGT GTT GAT GTT AGG GAT GGA AGA TTC

CAC AAC GGA AAC GCA ATA CAG TTG TGG CCA TGC AAG TCT AAT ACA GAT GCA

AAT CAG CTC TGG ACT TTG AAA AGA GAC AAT ACT ATT CGA TCT AAT GGA AAG

TGT TTA ACT ACT TAC GGG TAC AGT CCG GGA GTC TAT GTG ATG ATC TAT GAT

TGC AAT ACT GCT GCA ACT GAT GCC ACC CGC TGG CAA ATA TGG GAT

AAT GGA ACC ATC ATA AAT CCC AGA TCT AGT CTA GTT TTA GCA GCG ACA TCA

GGG AAC AGT GGT ACC ACA CTT ACG GTG CAA ACC AAC ATT TAT GCC GTT AGT

CAA GGT TGG CIT CCT ACT AAT AAT ACA CAA CCT TTT GTT ACA ACC ATT GTT

GGG CTA TAT GGT CTG TGC TTG CAA GCA AAT AGT GGA CAA GTA TGG ATA GAG

GAC TGT AGC AGT GAA AAG GCT GAA CAA CAG TGG GCT CTT TAT GCA GAT GGT

TCA ATA CGT CCT CAG CAA AAC CGA GAT AAT TGC CTT ACA AGT GAT TCT AAT

ATA CGG GAA ACA GTT GTT AAG ATC CTC TCT TGT GGC CCT GCA TCC TCT GGC

CAA CGA TGG ATG TTC AAG AAT GAT GGA ACC ATT TTA AAT TTG TAT AGT GGA

TTG GTG TTA GAT GTG AGG CGA TCG GAT CCG AGC CTT AAA CAA ATC ATT CTT

TAC CCT CTC CAT GGT GAC CCA AAC CAA ATA TGG TTA CCA TTA TTT

ou une séquence nucléotidique qui est équivalente à ladite séquence ou partie de celle-ci en raison de la dégénescence du code génétique.

5. Molécule d'ADN recombinant selon l'une quelconque des revendications précédentes, caractérisée en ce que ledit segment d'insertion a été introduit dans un vecteur de clonage qui est un plamide ou un bactériophage pour obtenir ladite molécule d'ADN recombinant.

6. Cellule hôte modifiée d'*E.Coli* ou de *Saccharomyces Cervisiae* contenant une molécule d'ADN recombinant selon l'une quelconque des revendications précédentes.

7. Microorganisme hôte modifié selon la revendication 6, lorsqu'elle est dépendante de la revendication 4, dans laquelle la cellule hôte est *E. coli,* caractérisé en ce que l vecteur de clonage est un plasmide choisi parmi pBR322, pAT153, pUC8, pGS15 ou pMB.

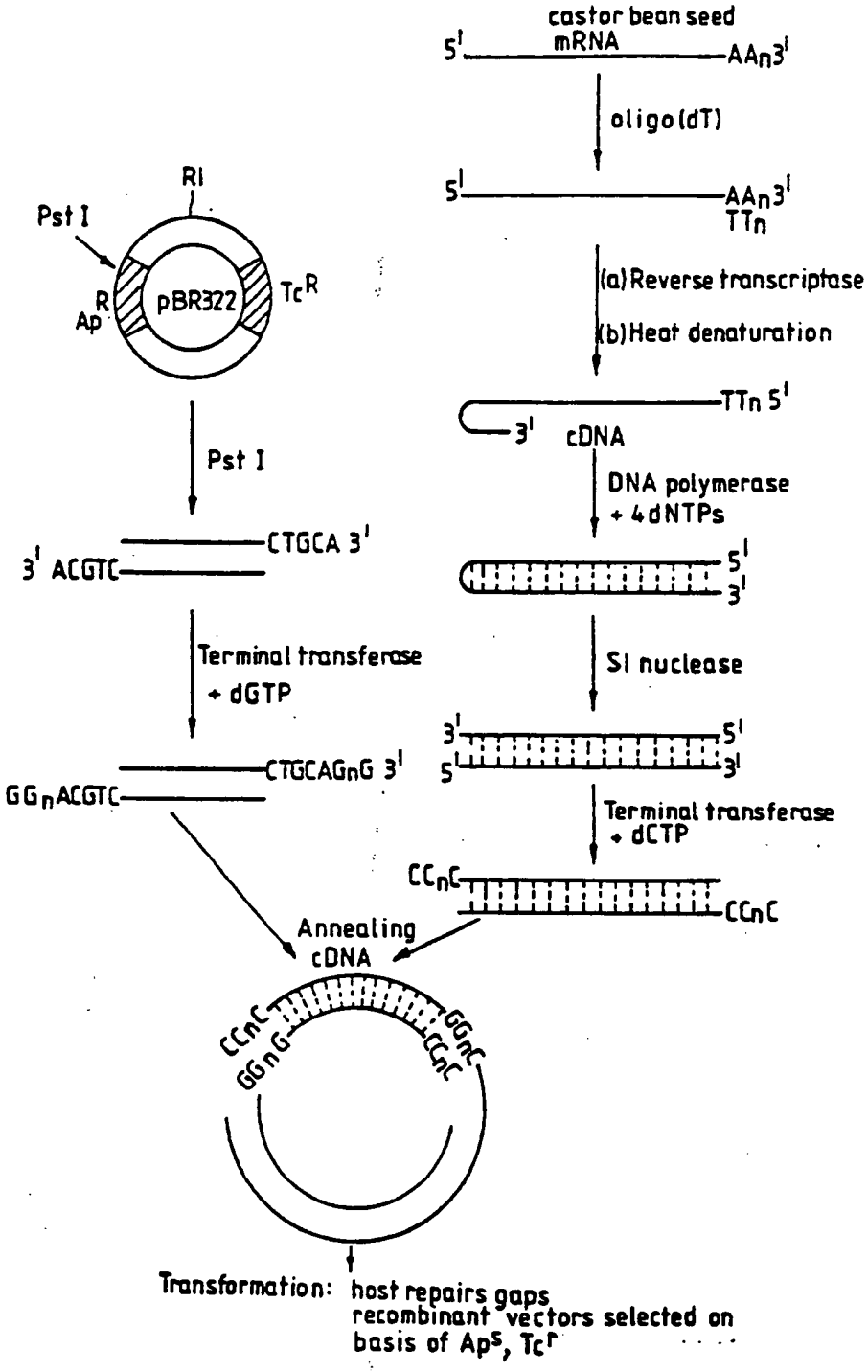
8. Microorganisme hôte m difié selon la revendication 6, lorsqu'elle st dépendante de la

revendication 4, dans laquelle la cellule hôte est *Sacchoromyces Cervisiae,* caractérisé en ce que le vecteur d clonage est pMa91, pMA230, YRp7, pLC544 et YEp6.

9. Procédé de préparation d'ADNc ayant une séquence nucléotidique telle que définie dans l'une quelconque des revendications 1 à 4, caractérisé en ce que l'on sépare à partir d'un mélange d'ARNm obtenu à partir de tissu de *Ricinus Communis* une fraction de poids moléculaire contenant l'ARNm codant pour la préproricine et en ce que l'on synthétise l'ADNc à partir de celui-ci par transcription inverse.

10. Procédé d'obtention d'une molécule d'ADN recombinant par insertion d'un ADNc double brin selon la revendication 8 dans un vecteur de clonage.

11. Procédé d'obtention d'un hôte génétiquement modifié d'*E. coli* ou *Sacchoromyces cervisiae,* caractérisé en ce que l'on introduit une molécule d'ADN recombinant selon la revendication 9 dans ledit hôte.


castor bean seed
mRNA
5' AAn 3'
oligo(dT)
5' AAn 3'
TTn
(a) Reverse transcriptase
(b) Heat denaturation
TTn 5'
3' cDNA
DNA polymerase
+ 4dNTPs
5'
3'
S1 nuclease
3'
5'
5'
3'
Terminal transferase
+ dCTP
CCnC
CCnC
RI
Pst I
R
Ap
pBR322
TcR
Pst I
CTGCA 3'
3' ACGTC
Terminal transferase
+ dGTP
CTGCAGnG 3'
GGnACGTC
Annealing
cDNA
CCnC
GGnG
GGnC
CCnC
Transformation: host repairs gaps
recombinant vectors selected on
basis of Ap^S, Tc^R